Development of a transient expression system for the alpha7 neuronal nicotinic acetylcholine receptor in mammalian cells

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Development of a Transient Expression System for the α7 Neuronal Nicotinic Acetylcholine Receptor in Mammalian Cells

Submitted by ROBERT CARLOS BARTON DAVY for the degree of

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To Mum and Dad

Above all, we are family

Abstract

Development of a Transient Expression System for the α7 Neuronal Nicotinic Acetylcholine Receptor in Mammalian Cells

The chick α7 neuronal nicotinic acetylcholine receptor (nAChR) was to be expressed transiently in the mammalian cell line and characterised using radioligand and fluorescence label binding assays. This expression system was then to be used to screen the responses of point mutated α7 nAChRs to methyllycaconitine (MLA). These results would then be used to determine the structural features of the receptor that make MLA such a potent and selective antagonist of the α7 nAChR.

COS-7 cells were transfected with α7 nAChR cDNA using a liposome mediated method. Low levels of specific [^{125}I]-αBgt binding (4.6 fmol/mg protein) was detected occasionally in these cells. Channel blocking antagonists, hexamethonium and *d*-Tubocurarine (dTC), were employed to determine their effect on the detectable levels of specific toxin binding.

When included in the post-transfection medium, 50μM hexamethonium increased the specific toxin binding to 10.7 fmol/mg protein and 10μM dTC to 14.8 fmol/mg protein. Transfected HEK-293 cells exhibited significantly greater levels of toxin binding than COS-7 cells, with a similar improvement in the presence of 10μM dTC (112.9 fmol/mg protein in the absence of dTC and 553.1 fmol/mg protein in the presence of dTC). Transfection with the Ca^{2+} impermeable chimaeric α7-5HT$_3$ receptor, showed

binding similar to that seen in the cells transfected in the presence of dTC. This possibly supports the Ca^{2+} blocking action of the antagonists, however the transfected cells do not suffer from decreases in total protein or viable cell number (usually associated with Ca^{2+} cytotoxicity). Thus the increases in specific toxin binding may be independent of Ca^{2+} influx.

MLA inhibited toxin binding in a saturable dose-dependent manner, with an IC_{50} of $\cong$1nM, confirming the presence of α7 nAChRs on the transfected cells. The FITC-αBgt immunofluorescence assay permitted the visualisation of the results recorded for the radioligand assay.

The potency of MLA was investigated by mutating amino acids in the ACh binding site of the α7 nAChR, to those in homologous positions in the MLA insensitive α1 containing nAChR. Preliminary data showed:

-The α7E-W mutant produced a similar response to nicotine as that seen for the wild-type.

-The α7K-W mutant was insensitive to MLA.

-The α7P-R mutant produced no response to nicotine.

The general conclusions from this study indicate that the presence of channel blocking antagonists leads to an increase in specific toxin binding in cells transfected by α7 nAChR cDNA.

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Abbreviations

αBgt	α-Bungarotoxin
ABT418	(s)-3-methyl-5-(1-methyl-2-pyrrolidinyl) isoxazole
ACh	Acetylcholine
AdMLP	Adenovirus major late promoter
mAChR(s)	Muscarinic acetylcholine receptors
nAChR(s)	Nicotinic acetylcholine receptor(s)
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BAPTA	1,2-bis(2-aminophenoxy)-ethane-N,N,N',N',-tetraacetic acid
BSA	Bovine serum albumin (fraction V)
(c)DNA	(Complementary) deoxyribonucleic acid
(c)RNA	(Complementary) ribonucleic acid
Cyt	Cytisine
[^{3}H]DDF	[^{3}H]-p-(dimethylamino)-benzenediazonium fluoroborate
DMPP	1,1-dimethyl-4-phenylpiperazinium
dTC	*d*-Tubocurarine
EC_{50}	Agonist concentration which gives half the maximal response
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N',-tetraacetic acid
FITC-αBgt	Fluorescein isothiocyanate conjugated to α-Bungarotoxin
GABA	γ-amino-butyric acid
$5HT_3$	5-hydroxytryptamine
IC_{50}	Ligand concentration which reduces the radioligand or functional responses to half the maximal
LGIC(s)	Ligand gated ion channel(s)
LTX-1	Lophotoxin-1
[^{3}H]-MBTA	4-(*N*-maleimido)benzyltri[^{3}H]-methyl-ammonium iodide
MLA	Methyllycaconitine
MOPS	3-(*N*-morpholino)propane-sulphonic acid
nBgt	Neuronal Bungarotoxin
NMDA	*N*-methyl-D-aspartate
NSTX	Neosurugatoxin
OXO-M	Oxotremorine M
PCR	Polymerase chain reaction
SDS	Sodium dodecylsulphate
TID	3-(trifluoromethyl)-3-m-([^{125}I]iodophenyl) diazirine
Tris	Tris(hydroxymethyl)methylamine
VDCC(s)	Voltage dependent calcium channel(s)

Chapter 1.

1. General Introduction

1.1. Neurotransmission and Receptor

The transduction of extracellular signals to the cytoplasm of a cell is, in the majority of cases, receptor mediated. For neurotransmitters, these receptors reside on the surface of the cell's plasma membrane. Exocytosis of neurotransmitter into the synaptic cleft, occurring within 1ms of the arrival of an action potential at the synaptic nerve terminal, is one of the fastest things an animal cell can do (Almers, 1994). The process by which a chemical transmitter message is decoded, by interaction with a specific recognition site on a receptor molecule, which in turn elicits a response from the cell, is termed neurotransmission (Eadie & Tyrer, 1983). One of the most extensively studied neurotransmitters is acetylcholine (ACh). The receptors for ACh fall into two broad classes, the muscarinic G protein linked receptors (mAChRs) and the nicotinic ligand gated ion channel receptors (nAChRs).

1.2. Ligand Gated Ion Channels

The nAChR belongs to a superfamily of receptors that includes $GABA_A$, glycine, serotonin $5HT_3$, sarcoplasmic reticulum ryanodine and glutamate receptors (Ortells & Lunt, 1995; Lees *et al.,* 1994; Maricq *et al.,* 1991; Stroud *et al.,* 1990; Miller, 1989). However, the glutamate activated ion channel family (AMPA, Kainate & NMDA) and ryanodine family of receptors are sufficiently structurally different from the rest of the LGIC superfamily to

warrant their own subclassifications (Bennett & Dingledine, 1995; Hollmann *et al.,* 1994; Seeburg, 1993; Unwin, 1993a; Zorzato *et al.,* 1990).

The nAChRs are themselves a family of receptors that can be further subdivided into 3 branches (Wonnacott, 1991, 1986):

1) The neuromuscular junction/Electroplaque (nj/e) nAChR.

2) Neuronal nAChRs that do not bind αBgt.

3) Neuronal nAChRs that do bind αBgt.

αBgt is an elapid curaremimetic neurotoxin isolated from the venom of *Bungarus multicinctus* This polypeptide nicotinic ligand competitively blocks (practically irreversibly) the nj/e type nAChR (Lentz & Wilson 1988; Sattelle *et al.,* 1983). nAChRs have been shown to be the major antigen in the muscle weakening autoimmune disease myasthenia gravis (Asher *et al.,* 1993; Souroujon *et al.,* 1993). Many brain acting drugs such as mecamylamine, amitriptyline, phencyclidine and trifluoperazine have the nAChR as their binding site (Connolly *et al.,* 1992).

Loss of [^{3}H]-acetylcholine and [^{3}H]-nicotine binding to nAChR in the brain is associated with loss of cognitive function (Lange *et al.,* 1993; Whitehouse *et al.,* 1986). In Alzheimer's disease, the loss of ligand binding seems to be accompanied by a decrease in the cerebral cortex activity of choline acetyltransferase and acetylcholinesterase; enzymes involved in acetylcholine synthesis and catabolism respectively (Flynn & March, 1986; Davis & Maloney, 1976). The cerebral cortex is also the area of the brain that contains the greatest density of neurofibrillary tangles (Goedert, 1987; Perry *et al.,* 1978), a characteristic histological marker for Alzheimer's disease. In

Parkinson's disease presynaptic nAChRs, present on the cell bodies and nerve terminals of the dopaminergic neurones of the nigrostriatal and mesolimbic systems, are lost (Prosad *et al.,* 1994; Grady *et al.,* 1992; Rowell *et al.,* 1987). Receptor loss within these systems is associated with Parkinsonian rigidity and tremor.

It is predicted that by the turn of the century at least 250 million premature deaths will have been caused by tobacco usage (Peto *et al.,* 1992). Nicotine is the addictive drug in tobacco (Balfour, 1994; Jaffe, 1990) and nicotine stimulation of dopamine release from mesocorticolimbic neurones, has been linked to the 'reward' pathways within the brain (Wise, 1980). This has led to the proposal by Wise and Bozarth (1987) of a 'psychomotor stimulant' theory of addiction. The theory states that the physical dependence on nicotine (from smoking or chewing) and other addictive substances (eg. alcohol, opiates, benzodiazepines, food, caffeine barbiturates and phencyclidine) is secondary to the activation of the 'reward' pathways through compulsive self administration. There is some recent evidence indicating that there may be a genetic predisposition component to addiction (Noble *et al.,* 1994). The anxiolytic effects of smoking may be due to nicotine decreasing the activity of hippocampal serotonergic systems (Benwell *et al.,* 1990).

Nicotine is known to improve performances in tests of attention, rapid information processing and other IQ correlates in man (Stough *et al.,* 1994; Jones *et al.,* 1992; Hindermarch *et al.,* 1980). Use of nicotinic antagonists impaired these cognitive abilities (Newhouse *et al.,* 1992). In

animals, nicotine improved learning and working memory (Hodges *et al.,* 1990; Harouturian *et al.,* 1985).

1.3. Neuromuscular Junction/Electroplaque nAChR

1.3.1. The Structure of the Receptor

The neuromuscular junction/electroplaque (nj/e) nAChR is the best characterised channel from the nAChR family. The electroplaque refers to the electric organs of *Torpedo*, *Electrophorus*, *Narke* and *Narcine* (Ishikawa *et al.,* 1980; Olsen *et al.,* 1973; Schmidt & Raftery, 1973, 1972) which are abundant sources of the nAChR (Conti-Tronconi & Raftery, 1982). The nj/e nAChR structure and function has been used as a model, on which most of the understanding of the other members of the nAChR family and LGIC superfamily has been based. The receptor can be considered to be an allosteric protein (Monod *et al.,* 1963), with 3 spatially distinct regions: a ligand binding region, a cation selective channel and an intracellular region that provides sites for the regulation of receptor activity. Despite being discrete, events at any one of the regions may affect the activity of the others.

The topography of the nj/e nAChR proposed by Noda *et al.,* (1983) and based on the transmembrane arrangements of bacteriorhodopsin and Glycophorin A, has held sway since the first subunits were cloned (refs. in Mishina *et al.,* 1985; Noda *et al.,* 1982; Sumikawa *et al.,* 1982)

1.3.2. The Subunits of the Receptor

The nAChR is composed of 4 homologous, but non-identical subunits in a pentameric arrangement (Lal & Yu, 1993; Changeux, 1990; Brisson & Unwin, 1985) (Figure 1.1). ie. two α-subunits and one of each β, γ and δ-subunits. However, in the mature neuromuscular junction, the 'foetal' γ-subunit is replaced by the 'adult' ε-subunit (Mishina *et al.,* 1986). Immunological characterisation of the receptor revealed that the α-subunits were not adjacent to each other (Fairclough *et al.,* 1983). The latest model proposes an arrangement of αβαγδ around the channel (Unwin, 1995). In *Torpedo*, the receptors are dimerised via disulphide bridges between the δ-subunits (McCrea *et al.,* 1987; Hamilton, 1979).

The general secondary structure is believed to be the same for all the subunits. Hydropathy analysis of subunit sequences, led to the belief that they were composed of 4 membrane spanning α-helices, termed M1 to M4 (Claudio *et al.,* 1983). Current thinking favours a 'mixed motif' model of α-helix and β-sheet (Ortells & Lunt, 1994). The evidence comes from direct electron density microscopy of frozen receptors and modelling based on bacterial B-pentamers (Unwin, 1995, 1993a; Stein *et al.,* 1992; Sixma *et al.,* 1991).

Using the nicotinic alkylating agent bromoacetyl-[^{3}H]-choline bromide, Momoi & Lennon (1982) showed that the α-subunit of the human neuromuscular junction nAChR was responsible for the binding of ACh. This confirmed the results of Haggerty & Froehner (1981) who demonstrated that [^{125}I]-αBgt only bound to the α-subunit of *Torpedo* electroplaque nAChR.

More recent work has shown that the γ and δ-subunits also contribute to the nicotinic ligand binding site (Sine *et al.,* 1994). They play a role in the maturation, regulation and cooperativity of the two α-subunit sites (Middleton & Cohen, 1991; Sine & Claudio, 1991a; Pedersen & Cohen, 1990). Other work has demonstrated that the αγ and αδ sites are non-equivalent, with the αγ site having a 100-300 fold greater affinity for nicotinic ligands than αδ site (Dunn *et al.,* 1993; Saedi *et al.,* 1991; Sine *et al.,* 1990). However, the nicotinic ligand binding site described by Unwin (1993b) in his electron density microscopy images are not located at the αγ and αδ interfaces. The ligand binding site will be discussed in more detail later.

The β-subunit does not play a role in the direct binding of nicotinic ligands. Its function seems to be to ensure efficient intracellular transport of the nascent nAChR, membrane clustering of the receptor and coordination of the cooperative mechanism of channel opening, after the binding of agonists to the α-subunits (Unwin, 1995; Wheeler *et al.,* 1994; Sumikawa & Gehle, 1992; Sine *et al.,* 1990).

1.3.3. The Ion Channel of the Receptor

When [^{3}H]-chlorpromazine, a nAChR non-competitive channel blocking drug, was used as a photoaffinity probe it labelled residues α-S248, β-S254, β-L257, γ-T253, γ-S257, γ-L260 and δ-S262 (Devillers-Thiery *et al.,* 1993). All these residues belong to the putative M2 helices of each subunit, with α-S248, β-S254, γ-S257 and δ-S262 occupying equivalent positions in their respective subunits. Other non-competitive affinity labelling agents such

as trimethylphosphonium and 3-(trifluoromethyl)-3-m-([^{125}I]iodophenyl) diazirine (TID) also labelled residues belonging exclusively to M2. Trimethylphosphonium labelled residues α-S248, β-S254 and δ-S262; (Hucho, 1986). TID labelled residues β-L257, β-V261, δ-L265 and δ-V269 in the absence of agonist, β-S250 and β-S254 are additionally labelled in the presence of agonist (White & Cohen, 1992). [^{3}H]-Meproadifen mustard labelled α-E262 at the C-terminal end of M2 (Pedersen *et al.,* 1992). However, [^{3}H]-quinacrine azide labelled residues at the N-terminal end of M1 (Dipaola *et al.,* 1990). This suggests that M1 plays some role in the lining of the ion channel and would also support an alternative channel model proposed by Akabas *et al.,* (1992).

Single channel conductance experiments performed on nAChRs with point mutations in the M2 region revealed the existence of rings of charged and polar amino acids (Imoto *et al.,* 1991, 1988). These rings have been shown to be responsible for the channel's ability to exclude anions and to distinguish between monovalent and divalent cations (Galzi *et al.,* 1992).

Most of the above evidence indicates that the M2 of each subunit lines the channel symmetrically and also that M2 has an helical secondary structure. These data agree with the Unwin, (1995) model that visualises M2 as a 'kinked' α-helix, with the kink proposed as the ion gate at the level of residue α-L251.

1.3.4. The ACh Binding Site

It had been originally thought that the disulphide bridged, 15 residue loop between α-C128 and α-C142 was the site of ACh binding, because this site was associated with αBgt binding (Mishina *et al.,* 1986). More recent work has shown that this loop, which is present in the subunits of nAChRs and other members of the LGIC superfamily (Betz, 1990; Stroud *et al.,* 1990) (Figure 1.2), does not form the site of ACh binding. It is, however, an essential requirement for correct subunit assembly and intracellular transport of the receptor (Sumikawa & Gehle, 1992; Verrall & Hall, 1992). Work on the loop from the $GABA_A$ receptor has produced similar results (Amin *et al.,* 1993).

Photoaffinity and alkylating agents such as [^{3}H]-DDF and [^{3}H]-MBTA have been used as competitive antagonists to identify those residues involved in nicotinic ligand binding. The residues labelled fell into three different regions of the α-subunit's N-terminal extracellular domain. In region A, W86 and Y93 were identified. In region B, W149 and Y151 were identified and in region C, Y190, Y198, C192 and C193 were identified (Galzi *et al.,* 1990; Dennis *et al.,* 1988; Kao *et al.,* 1984). This has given rise to the 'multiple loop' model for the ACh binding site, with the α-subunit contributing at least the 3 identified peptide loops (Galzi *et al.,* 1990, 1991a) (Figure 1.3). These observations were similar to those seen for the binding pocket of the glycine channel, with residues in positions almost homologous to Y93, W149 and Y198 being implicated (Schmieden *et al.,* 1992). Mutagenesis studies have shown that Y93, Y190 and Y198 are involved in the binding of the

quaternary N of ACh (Aylwin & White, 1994). Although their interactions are of different natures (Sine *et al.,* 1994), the use of aromatic residues in the nAChR binding site is similar to the 'aromatic gorge' feature seen in the ACh binding site of acetylcholinesterase (Sussman *et al.,* 1991).

Most of these labelled residues are conserved at homologous positions in all known α-subunits, except α5 (which has not been known to form a functional nAChR when expressed in *Xenopus* oocytes (Boulter *et al.,* 1990)) which lacks Y93 and Y190 and α8 which lacks Y151. In the model proposed by Unwin (1995), each subunit's N-terminal domain contains what appears to be three α-helical rods. From their positions, these α-helices form a cavity which is discernibly more open on the two α-subunits. The α-subunit cavities are non-identical in shape. These data, along with the recent identification of γ-W55 and δ-W57 involvement in the agonist binding site (Chiara & Cohen, 1992), may help to explain the difference in the pharmacologies seen between the sites.

The above labelling data, along with site directed mutagenesis established region α180-200 as contributing to the recognition of nicotinic ligands (Tomaselli *et al.,* 1991; Mishina *et al.,* 1985). This region includes the vicinal cyteines at 192 and 193 that are unique to α-subunits (Aplin & Wonnacott, 1994). They are involved in a rare *cis* half-cystinyl disulphide link that due to an energetic strain, can only exist in two conformations (Kellaris & Ware, 1989; Kao & Karlin, 1986; Thornton, 1981). The binding of agonist is believed to initiate a transition between the conformations. Thus, the vicinal

cysteines may be acting as a molecular switch that allosterically activates the receptor (Kao & Karlin, 1986).

1.4. Neuronal Nicotinic Acetylcholine Receptors That Do Not Bind αBgt

The subunits making up these nAChRs have been cloned through the use of PCR (Polymerase Chain Reaction) or low stringency screening of cDNA libraries with known subunit clones. The neuronal nAChRs appear to be composed of only two types of subunits, α and β/non-α (Nef *et al.,* 1988; Whiting & Lindstrom, 1987a, 1987b). β, is the generally accepted nomenclature for the second neuronal nAChR subunit. Affinity labelling experiments revealed that the nj/e nAChR nomenclature convention of naming subunits in order of increasing molecular weight, was incorrect for neuronal nAChRs (Whiting & Lindstrom, 1987a). The experiments showed that the ACh binding "β" subunit, had a greater molecular weight than the structural "α" subunit. The nomenclature was swapped (ie. α to β and β to α) when the N-terminal protein sequences of the subunits were compared to the sequences derived from the cloned subunits' cDNAs. The neuronal subunits are termed retrospectively, α2-9 and β2-4 (ie. the nj/e nAChR α and β subunits termed α1 and β1). Non αBgt binding neuronal nAChRs subunits have been isolated from species as diverse as human, rat, chicken and *Drosophila* (Sargent, 1993).

The α-subunits have high amino acid homology with one another (50%-70%). The β-subunits are as different from each other as they

are from α-subunits (30%-40%). However, β-subunit genes with the same names from different species are highly homologous (>70%). Hydropathy analysis of the neuronal nAChR subunit sequences indicate that they share the same overall topography, as the nj/e nAChR subunits (refs. in Sargent, 1993). *In vitro* expression studies using α2, α3 and α4 heterologously coexpressed with either β2 or β4, showed that they formed functional nAChR that were not blocked by αBgt. This situation may be a simplified one, since the proposal of the existence of native nAChRs composed of different α-subunits (eg. one receptor containing α3 and α5 as the ACh binding subunits; Conroy *et al.,* 1992; Listerud *et al.,* 1991). With this number of subunits, there is the potential for many different forms of neuronal nAChR to exist.

In brain, the predominately expressed subtype of nAChR is composed of α4β2 (Whiting & Lindstrom, 1988). It does not bind αBgt and has a high affinity for nicotine. This nAChR subtype is believed to be involved in many of the presynaptic ACh activated pathways mentioned above, and in preterminal modulation of GABAergic axons in rat Interperduncular Nucleus (Lena *et al.,* 1993). The α4β2 nAChR has been identified as the site of action of ABT418, a novel nicotinic agonist that, in animal models of the human condition of Alzheimer's disease, treats the cognitive, physical and emotional dysfunctions (Arneric *et al.,* 1994; Decker *et al.,* 1994). The analgesic agent (±)-Epibatidine, a natural product isolated from the venom of the poison arrow frog *Epidedobates tricolor*, binds potently (K_i 43pM) to α4β2 nAChRs present in rat membranes (Sullivan *et al.,* 1994). Upregulation of the α4β2 receptor subtype in the brain is a characteristic of addiction to or chronic

administration of nicotine (Flores *et al.,* 1992). Chronic administration of nicotine to *Xenopus* oocytes and cultured cells expressing $\alpha4\beta2$ receptor subtype also results in upregulation (Peng *et al.,* 1994). The observed increase in B_{max} (receptor density), no change in K_d (affinity) and decrease in receptor function (Marks *et al.,* 1983) in the brain of animals chronically treated with nicotine is paradoxical, since chronic administration of agonist to other receptors (eg. hormone, catecholamine and GABA) normally leads to a down regulation of the target receptors (Wonnacott, 1990; Maloteaux *et al.,* 1987).

Immunological characterisation and channel conductance analysis of $\alpha4\beta2$ receptors containing mutant subunits, showed the composition to be $(\alpha4)_2(\beta2)_3$ (Anand *et al.,* 1991; Cooper *et al.,* 1991). Thus neuronal nAChR may possess the same pentameric pseudosymmetric quaternary structure as the nj/e nAChR. $\alpha3/\alpha5/\beta4$ containing receptors are thought to be the primary postsynaptic subtype of nAChR found in ganglionic material (Corriveau & Berg, 1993). Receptors composed of these subunits do not bind αBgt. Table 1.1 shows the distribution and abundance of the subunits within the mammalian CNS.

One problem faced by neuronal nAChR researchers, has been the lack of receptor subtype specific ligands (eg. ABT418 for the $\alpha4\beta2$ nAChR subtype). This problem has been overcome, to some extent, by the use of a panel of nicotinic ligands in order to identify specific nAChR subtypes. nAChRs formed from $\alpha2$, $\alpha3$, or $\alpha4$ subunits in pairwise combinations with $\beta2$ or $\beta4$, were expressed in *Xenopus* oocytes. Using

cytisine, nicotine, ACh and DMPP (1,1-dimethyl-4-phenylpiperazinium) as nicotinic agonists, Luetje & Patrick (1991) showed that each receptor subtype had an unique rank order of potency (Table 1.2). These results demonstrated that both α and β subunits played a role in determining nicotinic ligand potency. Receptor subtypes containing $\beta 2$ were almost completely insensitive to cytisine, whereas in $\beta 4$ containing receptor subtypes this ligand was the most potent.

Neurotoxin antagonists such as nBgt (neuronal Bungarotoxin, isolated as a fraction of *Bungarus multicintus* venom), NSTX (neosurugatoxin, isolated from *Babylonica japonica*) and LTX-1 (lophotoxin-1, a naturally occurring analog of lophotoxin isolated from the sea whip *Lophogorgia rigida*) have also been employed to discriminate between *Xenopus* oocyte expressed receptor subtypes (Luetje *et al.,* 1990; Duvoisin *et al.,* 1989). These neurotoxins also demonstrated the involvement of both the α and β subunits in determining receptor ligand affinity (Table 1.2). Methods such as open channel conductances and channel gating "open-time" distributions have been used to distinguish between some of the neuronal nAChR subtypes (Papke & Heinemann, 1991; Papke *et al.,* 1989). However, research continues to isolate from natural sources or synthesise based on binding site data, neuronal nAChR subtype specific ligands.

1.5. Neuronal Nicotinic Receptors That Do Bind αBgt

1.5.1. The Subunits

Until 5 years ago, the physiological significance of αBgt binding sites in the CNS was widely questioned (Clarke, 1992). The αBgt binding sites in the brain displayed a pharmacological and biochemical profile similar to the nj/e nAChR (Morley & Kemp, 1981; Oswald & Freeman, 1981), but αBgt's inability to attenuate brain nicotinic transmissions highlighted a distinct difference (Chiappinelli, 1985; Morley & Kemp, 1981). The confirmation that the αBgt binding sites were actually on neuronal nAChRs came with the molecular cloning and expression of these receptor subunits. Recently, Zhang *et al.,* (1994) and Vijayaraghavan *et al.,* (1992) have shown electrophysiologically that neuronal nAChRs that bind αBgt function as LGICs.

To date 3 cDNA's, α7, α8 and α9, have been cloned from neuronal nAChRs that bind αBgt (Elgoyhen *et al.,* 1994; Couturier *et al.,* 1990a; Schoepfer *et al.,* 1990). Their sequence homologies (Table 1.3) indicate that these subunits are clearly members of the nAChR family, with all the general architectural features of agonist binding subunits (eg. N-terminal extracellular cysteine loops and vicinal cysteines). However, the relatively low sequence homology between α7, α8 and α1, and α7, α8 and α9 indicates that the these 3 subunits belong to two groups. One group contains α7 and α8, and the other group contains α9, with both these groups diverging slightly from the main phylogenic group containing the other nAChR family subunits. Analysis of the intron-exon boundaries positions reveal several differences

between the α7 and α9 genes, and the genes of the other nAChR family subunits (Elgoyhen *et al.,* 1994; Boulter *et al.,* 1990; Couturier *et al.,* 1990a; Buonanno *et al.,* 1989; Nef *et al.,* 1988). The gene structure of α8 is as yet unknown, since it has only been isolated as a cDNA. The human α7 gene is located on chromosome 15 (Chini *et al.,* 1994) and the mouse α7 gene on chromosome 9 (Anand & Lindstrom, 1992). The chromosomal loci of chick α8 and rat α9 are as yet unknown.

1.5.2. The Pharmacology of Neuronal Nicotinic Receptors That Bind αBgt

An important feature that sets the α7, α8 and α9 subunits apart from the other nAChR family subunits is their ability to form functional homomeric receptors when expressed alone in *Xenopus* oocytes (Elgoyhen *et al.,* 1994; Couturier *et al.,* 1990a; Schoepfer *et al.,* 1990). Homomeric, functional expression in *Xenopus* oocytes has been reported for $GABA_A$, glycine and $5HT_3$ receptor subunits (Green *et al.,* 1995; Schmieden *et al.,* 1989; Blair *et al.,* 1988). This ability to form homomeric receptors is also shared by the invertebrate nAChR subunit ARL2, isolated from the locust *Schistocerca gregaria* (Marshall *et al.,* 1990). Homomeric ARL2 receptors also bind αBgt (Amar *et al.,* 1992). Expression of homomeric rat α4 receptors in *Xenopus* oocytes has been reported, but these receptors gave very weak responses even to 100μM ACh (Boulter *et al.,* 1987).

1.5.2.1. The α7 and α8 Containing nAChRs

Keyser *et al.,* (1993) showed that chick retina contained 3 subtypes of nAChR that bound αBgt:

1) A neuronal nAChR containing α7

2) A neuronal nAChR containing α7 and α8

3) A neuronal nAChR containing α8

These three receptor subtypes were also found in chick brain, but in different proportions. In retina, the α8 containing nAChR predominated, accounting for ≅69% of αBgt binding. The remaining αBgt binding in the retina could be attributed to the α7 (14%) and α7/α8 (17%) nAChRs. The α7 containing nAChR predominated in brain, accounting for ≅75% of αBgt binding. The α8 (9%) and α7/α8 (15%) nAChRs accounted for the remaining binding. However, it should be noted that only 80-90% of the total αBgt binding could be precipitated from the various tissue extracts. The ≅10% unaccounted αBgt binding may indicate the existence of as yet unknown members of the neuronal αBgt binding group (Gotti *et al.,* 1994).

Sucrose density gradients indicate that the α7 homomeric nAChR is a pentamer of α7 subunits, like the nj/e nAChR (Anand *et al.,* 1993a). It is assumed that the homomeric α8 nAChR is also a pentamer of α8 subunits. Although there are many similarities in pharmacology between the homomeric α7 and α8 nAChRs and the native α7, α8 and α7/α8 nAChRs, the presence of homomeric neuronal α7 and α8 nAChRs seems unlikely. The immunoisolated native α7 and α7/α8 nAChRs both contained an

uncharacterised M_r 52,000 protein (Gotti *et al.,* 1994). This protein may influence the pharmacological properties of the native receptors.

Table 1.4a shows some of the pharmacological characteristics of the homomeric and native α7 and α8 containing nAChRs. Native α8 nAChRs exhibit two classes of binding site, an high affinity site and a low affinity site. The native α8 high affinity site has a significantly higher affinity for most nicotinic ligands than native α7 nAChRs (Gerzanich *et al.,* 1994; Anand *et al.,* 1993b). The homomeric α8 nAChR exhibits only a single binding site. The pharmacology of this binding site for nicotinic ligands, lies in between the high and low affinity binding sites on the native α8 nAChR. Both homomeric and native α7 and α8 nAChRs are blocked by atropine, a classical mAChR antagonist and strychnine, a classical glycine receptor antagonist. Both α7 and α8 homomeric nAChRs are virtually irreversibly blocked by αBgt and nBgt.

1.5.2.2. The α9 Containing nAChRs

The pharmacology of the α9 nAChR is the most enigmatic of the homomeric nAChRs. When expressed in *Xenopus* oocytes, homomeric α9 nAChRs respond to ACh with an EC_{50} of 10μM. This puts its affinity for ACh in between homomeric α7 (320μM) and α8 (1.9μM) nAChRs. However, it does not respond to the classical nAChR agonists nicotine and cytisine (Elgoyhen *et al.,* 1994). Table 1.4b shows the pharmacology of the homomeric α9 nAChR. The nicotinic agonist DMPP elicits a small response from homomeric α9 nAChRs, similar to that seen for the homomeric α7

nAChR where it is a partial agonist (Amar *et al.,* 1993; Séguéla *et al.,* 1993). The mAChR agonist OXO-M (Oxotremorine-M), also elicited a small response. The homomeric α9 nAChR's binding of agonists thus exhibits a mixed nicotinic-muscarinic pharmacological profile. When the antagonists are considered, this mixed pharmacological profile is confirmed.

Nicotine and muscarine, both agonists amongst their family of receptors, block ACh responses, indicating that these ligands bind to the homomeric α9 nAChR but do not activate the channel. ACh responses were blocked by dTC (*d*-Tubocurarine) and atropine. Strychnine potently blocked ACh responses, with an IC_{50} of 20nM. This indicates that the ligand binding site on the homomeric α9 nAChR may also possess some glycinergic characteristics. Whereas homomeric α7 and α8 nAChRs are virtually irreversibly blocked by αBgt and nBgt, the homomeric α9 nAChR was only transiently blocked even by 100nM concentrations of these toxins.

The pharmacology of the homomeric α9 nAChR is similar to the native nAChR identified on chick cochlea hair cells (Fuchs & Murrow, 1992). This nAChR is reversibly blocked by αBgt, not activated by nicotine, equipotently antagonised by curare and atropine and potently antagonised by strychnine. Similar nAChRs have been described on the cochlea outer hair cells of cats and guinea pigs (Housley & Ashmore, 1991; Fex & Adams, 1978). *In situ* hybridisation of α9 cRNA to rat embryos and adult rat brain found no evidence of α9 gene expression in the CNS (Elgoyhen *et al.,* 1994). However, α9 gene expression was detected in the pituitary, embryo tongue,

embryo sternohyoid muscle, nasal epithelium, inner and outer cochlea hair cells.

1.5.3. Ca^{2+} Permeability and Desensitisation of Neuronal nAChRs That Bind αBgt.

As well as gating Na^+, neuronal nAChRs are very permeable to Ca^{2+} (Sargent, 1993; Vernino *et al.,* 1992). This also true of *Xenopus* oocyte expressed homomeric α7, α8 and α9 nAChRs (Elgoyhen *et al.,* 1994; Gerzanich *et al.,* 1994; Bertrand *et al.,* 1993; Galzi *et al.,* 1992). The permeability ratio of Ca^{2+}:Na^+ (P_{Ca}:P_{Na}) for nj/e nAChRs is between 0.1 to 0.3 (Dwyer *et al.,* 1980). A P_{Ca}:P_{Na} value of 20 has been reported for the rat homomeric α7 nAChR (Seguela *et al.,* 1993). This is greater than the P_{Ca}:P_{Na} value of 10.6 reported for the NMDA glutamate receptor (Mayer & Westbrook, 1992), a receptor regarded as one of the main pathways of promoting neuronal Ca^{2+} influx (Mulle *et al.,* 1992). It is believed that both α8 and α9 nAChRs share a Ca^{2+} permeability similar to α7 (Elgoyhen *et al.,* 1994; Gerzanich *et al.,* 1994). Thus, a substantial fraction of the current carried by these homomeric receptors is carried by Ca^{2+}.

Ca^{2+}-dependent Cl^- channels have been identified in *Xenopus* oocytes (Boton *et al.,* 1989). The use of Ca^{2+} free solutions, external and internal Ca^{2+} chelators such as EGTA and BAPTA, Ca^{2+}-dependent Cl^- channel blockers such as Niflumic and Flufenamic acid (White & Aylwin, 1990) have demonstrated that *Xenopus* oocyte expressed nAChR responses are heavily contributed to by secondary activation of Ca^{2+}-dependent Cl^-

channels (Elgoyhen *et al.,* 1994; Gerzanich *et al.,* 1994; Amar *et al.,* 1993; Seguela *et al.,* 1993). In single channel experiments, Ca^{2+} is known to directly allosterically modulate native nAChRs by increasing the frequency of channel opening without changing the duration of channel opening (Vernino *et al.,* 1992). Ca^{2+} may also indirectly modulate neuronal nAChR action by setting in motion an enzyme cascade, ending with the desensitisation of the nAChR through its phosphorylation (Swope *et al.,* 1992; Huganir & Greengard, 1990; Huganir *et al.,* 1986).

Desensitised nAChRs are capable of binding nicotinic ligands with an increased affinity than in their resting state, but are unable to activate the channel (Lena & Changeux, 1993). Figure 1.4 shows a current model of the allosteric conformations and transitions of a nAChR. The nAChR can exist in several interconvertible states. R is the nAChR in a resting state. A is the nAChR in the active state with agonist bound and channel activated. D represents one of several desensitised states in which the channel is closed, but the affinity for agonist is increased. The change in affinity of the D states was predicted by Monod *et al.,* (1965) in their 'Concerted' model of allostery. This model explains some of the data seen when comparing nAChR functional and binding EC_{50}'s. Binding assays are usually carried out under conditions where the nAChRs are in a desensitised state. The difference in functional EC_{50} (ie. the R states affinity for agonist (Stephens, 1994)) and binding EC_{50} may vary by as much as 1-2 orders of magnitude (Gerzanich *et al.,* 1994). Site directed mutagenesis on the M2 of homomeric $\alpha 7$ nAChRs produced a mutant nAChR with a decreased rate of desensitisation, but with

an EC_{50} more than 2 orders of magnitude more sensitive for ACh (Revah *et al.,* 1991). This was probably due to the mutation enabling one of the desensitised states to activate the channel (Bertrand *et al.,* 1993; Galzi *et al.,* 1992). However, this model cannot fully explain the mechanisms behind mutants with multiple conductance states or mutations permitting antagonist to function as agonists (Bertrand *et al.,* 1992).

1.5.4. The Role of Native Neuronal nAChRs That Bind αBgt.

In chick optic tectum expression of α7 containing nAChRs is developmentally regulated between stages E5 to E16 (embryonic days 5 to 16). The level of α7 mRNA increases from E5 and peaks around E12. This coincides with the neurogenesis of connections between the optic tectum and the brain stem nuclei (Couturier *et al.,* 1990a). Optic tectum α7 mRNA levels from E10 onwards correlate with levels of αBgt binding (Couturier *et al.,* 1990a; Wang & Schmidt, 1976). From neonate to adult, αBgt mRNA levels in optic tectum decrease to a low plateau; this coincides with the maturation of cholinergic synapses (Wang & Schmidt, 1976).

The permeability to Ca^{2+} of neuronal nAChRs that bind αBgt has been discussed above. The non-synaptic α7 containing nAChRs of chick ciliary ganglion are capable of elevating internal Ca^{2+} levels without activating VDCCs (voltage dependent calcium channels) (Rathouz & Berg, 1994; Vijayaraghavan *et al.,* 1992). Hence, local Ca^{2+} dependent events may occur within a neurone without the need for depolarisation of the membrane. This is similar to the presynaptic neuronal nAChRs described by Wonnacott *et al.,*

(1990) that could directly trigger neurotransmitter release, through Ca^{2+} influx, without the need for depolarisation of the terminal. It should be noted that Ca^{2+} plays a fundamental role in the exocytosis of synaptic vesicles (Rubin, 1974).

Hippocampal α7 containing nAChRs have been recently pharmacologically and electrophysiologically characterised (Barrantes *et al.,* 1995, 1994; Alkondon *et al.,* 1994; Alkondon & Albuquerque, 1993, 1991). Blockade of these nAChRs with αBgt led to an increase in the mRNA for the neurotrophic factors NGF (nerve growth factor) and BDGF (brain derived growth factor) in discrete areas of the hippocampus (Freedman *et al.,* 1993). These hippocampal nAChRs are also involved in the gating response to repeated auditory stimuli (Luntz-Leyman *et al.,* 1992), the dysfunction of which may be associated with the auditory hallucinations experienced by some schizophrenia suffers (Freedman *et al.,* 1991). Rearrangements around the chromosomal locus of the human α7 gene are associated with mild mental retardation and some treatment-resistant epilepsies (Chini *et al.,* 1993). This condition is mirrored in a strain of seizure susceptible mouse with an high density of hippocampal αBgt binding nAChRs. It should be remembered that α8 containing nAChRs are also present in the brain (Keyser *et al.,* 1993; Schoepfer *et al.,* 1990). Hence, some of the brain functions described above may also involve α8 containing nAChRs.

In vitro culture of retina cells in presence of ACh led to a decrease in the number of new neurite processes, when compared to the control (Lipton *et al.,* 1988). The reverse was seen when the nAChRs were

blocked with dTC. This mechanism may involve α8 containing nAChRs which are the predominant αBgt binding nAChR subtype in the retina. *In vitro* exposure of cultured ciliary ganglion to nicotine, where α7 containing nAChRs are present on the neurites and cell bodies (Ravdin *et al.,* 1981), causes neurite retraction of up to 35% of its length, when compared to the control (Pugh & Berg, 1994). Blockade of these nAChRs with αBgt or dTC prevented neurite retraction. All these neurotrophic effects seem to involve increasing internal Ca^{2+} levels through the activation of VDCCs (Pugh & Berg, 1994). These data indicate that α7 and α8 containing nAChRs may have a role in neural plasticity by modulating synapse remodelling (Lipton & Kater, 1989).

The role of α9 containing nAChRs in tongue and stenohyoid muscle is unknown, since α9 transcripts seem to be absent in all other skeletal muscles (Elgoyhen *et al.,* 1994). The presence of the α9 transcript in pituitary (pars tuberalis) may indicate cholinergic involvement in some endocrine effects, such as the coordination of pineal hormone and melotonin in circadian systems (Wittkowski *et al.,* 1992; Cassone, 1990). Cholinergic modulation has been implicated in olfactory receptor neurone potentials (Bouvet *et al.,* 1988). This may involve α9, due its location within the nasal epithelium. In cochlea, the location of α9 transcripts in outer hair cells indicate that there may be cholinergic involvement in the encoding of auditory stimuli and protection against acoustic trauma (Elgoyhen *et al.,* 1994; Rajan & Johnstone, 1988).

Neuronal nAChRs that bind αBgt have been identified as functional nAChRs that may play many diverse physiological roles, the mechanisms of which are not fully understood.

1.6. Project Aims

Using the nAChR family as models, much of our understanding of LGIC structure and function has been inferred through their study. Such data indicates that the majority of LGICs are pentameric complexes. The subunits forming these complexes seem to share the same overall transmembrane topography and architectural features (exceptions to this have been mentioned above). The nAChR has been used as a model allosteric protein. It demonstrates excellently how events occurring at spatially distinct binding sites are coordinated, through mechanisms not fully understood, over the quaternary structure of the protein to gate the ion channel. Reconstituted channel conductances, patch clamp, immunological characterisation, radioligand binding and affinity ligand binding studies on the nAChR have revealed some details about the structure and function LGICs.

Research to highlight the key residues involved in nicotine ligand binding and hence determine the structure of the ACh binding site, has been one of the main goals of the last 10 years. The structural similarities between the subunits of the LGIC superfamily, suggests that these key residues may be in equivalent positions in the ligand binding subunits across the superfamily. Knowledge of the binding site structure would in turn feedback into better receptor specific pharmacophore design. A

pharmacophore is defined as the "specific three dimensional arrangement of essential chemical groups common to active molecules, that is recognised by a single receptor" (Sheridan *et al.,* 1986). Refinement of the pharmacophore model would lead to the design of new ligands to probe the binding site further.

Detailed X-ray diffraction or electron microscopy visualisation of the binding site is still some time off (Unwin, 1995; Brisson & Unwin, 1984), so ligand binding and site directed mutagenesis are still the best tools for investigating the ACh binding site's structure. The nj/e nAChR, due to its availability in a rich and convenient source, has been the subject of most research in the past (Fuchs *et al.,* 1984). The study of the neuronal nAChRs has been more problematic because of their low abundance in the tissues in which they are found. Cloning of the neuronal nAChRs' subunits, along with suitable expression systems, has meant that these receptors can now be studied without the need to purify them from their native tissues.

This project will look at the functional homomeric channel formed by the chick $\alpha 7$ nAChR subunit. The ability to form homomeric channels has been exploited when investigating the nature of the charged and polar rings flanking the ion channel and the position of the 'ion gate' (Galzi *et al.,* 1992). Any mutation introduced would be in all the subunits making up the receptor channel. The *Xenopus* oocyte expression system has been used for many years to study the expression, function and electrophysiology of LGICs (Kay & Peng, 1991). However, this system is limited. Proteins are produced only during the lifetime of the injected RNA or

DNA, hence studies requiring LGICs to be expressed for more than a few days are not practical. Since each oocyte must be individually injected, only small quantities of LGICs can be produced. Transfecting a cultured cell line either transiently or stably with LGICs may be the answer. A transfected cultured cell line would provide large numbers of identical cells expressing LGICs that are biochemically, pharmacologically and functionally similar to those in the cells from which the cDNAs were derived. Subsequently, if mutants LGICs were designed and expressed, this system would permit the results to be more easily interpreted and explained, with reference to the native LGICs.

Methyllycaconitine (MLA) is a potent inhibitor of ACh and anatoxin-a binding at the homomeric α7 nAChR (Thomas *et al.,* 1993; Wonnacott *et al.,* 1993). Although ACh and anatoxin-a are agonists and MLA an antagonist, they must possess a similar pharmacophore in order to compete for the same binding site. Hence, data about the key residues involved in the binding of MLA could help in the understanding of how agonists are bound.

The project was in two parts. 1) To develop a transient expression system in a mammalian cell line for the homomeric α7 nAChR. This nAChR would then be characterised through the use of radioligand and fluorescence labelled binding assays. 2) To explore, through site directed mutagenesis, the structural features in the homomeric α7 nAChR critical to the potency of the competitive agonist MLA. This would be achieved by

exploiting the transient expression system, developed above, to evaluate the mutants.

Figure 1.1

a) Table of K values for α, β, γ and δ-subunits from *Torpedo californica*. The evolutionary relationships among the subunits can be determined by, comparing the aligned amino acid sequences and calculating the amino acid difference per residue for each pair of mature subunits. K is defined as K=M/N, where M is the number of positions at which different amino acids are found between the sequences compared, and N the total number of positions (modified from Noda *et al.,* 1983). Therefore, the lower the value of K the higher the homology between the sequences.

b) Phylogenic tree of the 4 *Torpedo* subunits. Tree was generated based on information from K values (Miyata & Hayashida, 1982), with t_0, t_1 and t_2 as the relative dates of branching in years from the ancestral subunits α_0, α_1, and α_2.

$t_1=0.82t_0$

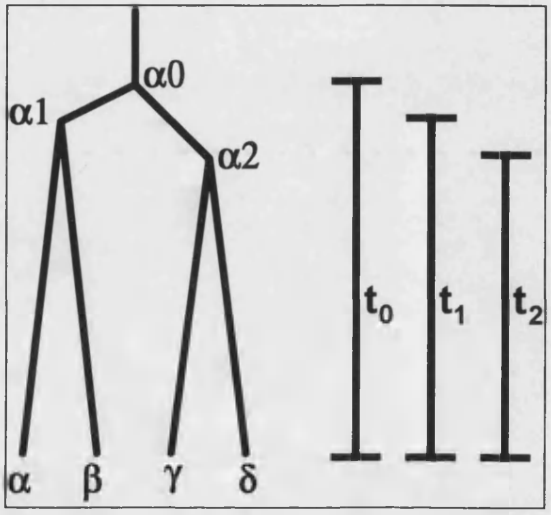
$t_2=0.65t_0$

c) Features of the nAChR. The γ/ε subunit has been removed for ease of viewing. The homologous subunits are arranged in a ring and the central axis of this ring makes a pathway (pore) for the ions. The bulk of the protein protrudes into synaptic cleft and contains the neurotransmitter binding pocket. Charged groups lining the pore screen out wrongly charged ions and facilitate the flow of correctly charged ions (data from Unwin, 1995, 1993a)

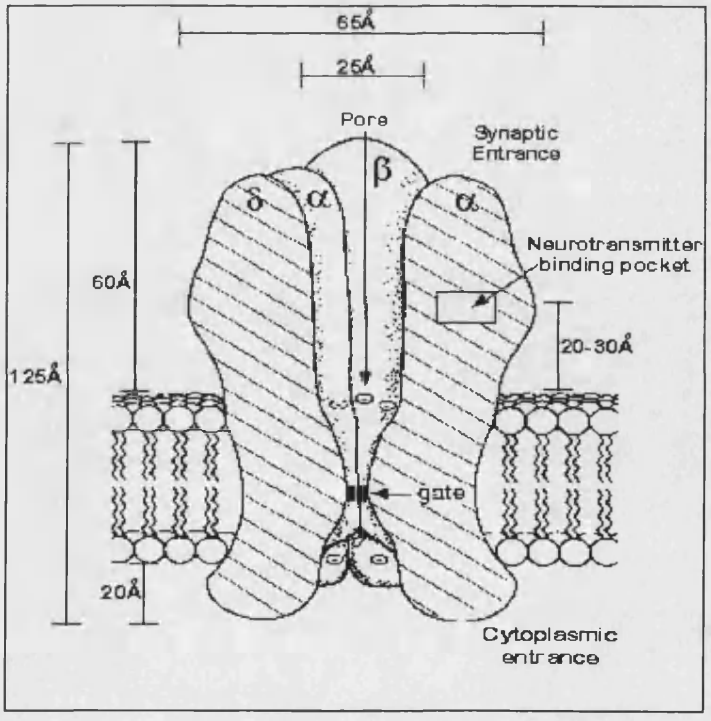
a)

	α	β	γ	δ
α	0.00	0.57	0.64	0.63
β		0.00	0.58	0.59
γ			0.00	0.43
δ				0.00

b)



c)



Human α1	**C**	E	I	I	V	T	H	**F**	**P**	**F**	**D**	Q	Q	**N**	**C**
Torpedo α1	**C**	P	I	A	V	T	Y	**F**	**P**	**F**	**D**	W	Q	**N**	**C**
Chick α1	**C**	E	I	I	V	T	Y	**F**	**P**	**F**	**D**	Q	Q	**N**	**C**
Mouse α1	**C**	E	I	I	V	T	H	**F**	**P**	**F**	**D**	E	Q	**N**	**C**
Rat α1	**C**	E	T	I	V	T	H	**F**	**P**	**F**	**D**	E	Q	**N**	**C**
Chick α2	**C**	S	I	D	V	T	Y	**F**	**P**	**F**	**D**	Q	Q	**N**	**C**
Chick α3	**C**	K	I	D	V	T	Y	**F**	**P**	**F**	**D**	Y	Q	**N**	**C**
Chick α4	**C**	S	I	D	V	T	Y	**F**	**P**	**F**	**D**	Q	Q	**N**	**C**
Chick α5	**C**	T	I	D	V	T	F	**F**	**P**	**F**	**D**	L	Q	**N**	**C**
Rat α6	**C**	P	M	D	I	T	F	**F**	**P**	**F**	**D**	H	Q	**N**	**C**
Chick α7	**C**	Y	I	D	V	R	W	**F**	**P**	**F**	**D**	V	Q	K	**C**
Chick α8	**C**	Y	I	D	V	R	W	**F**	**P**	**F**	**D**	V	Q	K	**C**
Rat α9	**C**	V	V	D	V	T	Y	**F**	**P**	**F**	**D**	S	Q	Q	**C**
Torpedo β1	**C**	T	I	K	V	M	Y	**F**	**P**	**F**	**D**	W	Q	**N**	**C**
Rat β1	**C**	S	I	Q	V	T	Y	**F**	**P**	**F**	**D**	W	Q	**N**	**C**
Chick β2	**C**	K	I	E	V	K	H	**F**	**P**	**F**	**D**	Q	Q	**N**	**C**
Rat β3	**C**	T	M	D	V	T	F	**F**	**P**	**F**	**D**	R	Q	**N**	**C**
Chick β4	**C**	K	I	E	V	K	H	**F**	**P**	**F**	**D**	Q	Q	**N**	**C**
Torpedo γ	**C**	P	I	A	V	T	Y	**F**	**P**	**F**	**D**	W	Q	**N**	**C**
Torpedo δ	**C**	P	I	N	V	L	Y	**F**	**P**	**F**	**D**	W	Q	**N**	**C**
Rat ε	**C**	Y	I	D	V	R	W	**F**	**P**	**F**	**D**	V	Q	K	**C**
ARL2 (Locust)	**C**	E	I	D	V	E	Y	**F**	**P**	**F**	**D**	E	Q	T	**C**
ALS (*Drosophila*)	**C**	T	I	D	V	T	Y	**F**	**P**	**F**	**D**	Q	Q	T	**C**
GABA-α	**C**	P	M	H	L	E	D	**F**	**P**	M	**D**	A	H	A	**C**
GABA-β	**C**	M	M	D	L	R	R	Y	**P**	L	**D**	E	Q	**N**	**C**
Glycine (48K)	**C**	P	M	D	L	K	N	**F**	**P**	M	**D**	V	Q	T	**C**
$5HT_3$ (Serotonin)	**C**	S	L	D	I	Y	N	**F**	**P**	**F**	**D**	V	Q	**N**	**C**
Glutamate (GluR1)*	**C**	F	I	T	S	P	-	**F**	**P**	V	**D**	T	S	**N**	Q

Figure 1.2

Alignments of conserved cysteine loops from the LGIC superfamily. The most conserved residues are highlighted in bold.

* - From the glutamate receptor family, this GluR1 sequence is the most homologous to the cysteine loop sequences seen in the other members of the LGIC superfamily (refs in Hollmann & Heinemann, 1994). The glutamate receptor subunits lack the conserved cysteine loop, thus reinforcing the case for their own subclassification within the LGIC superfamily.

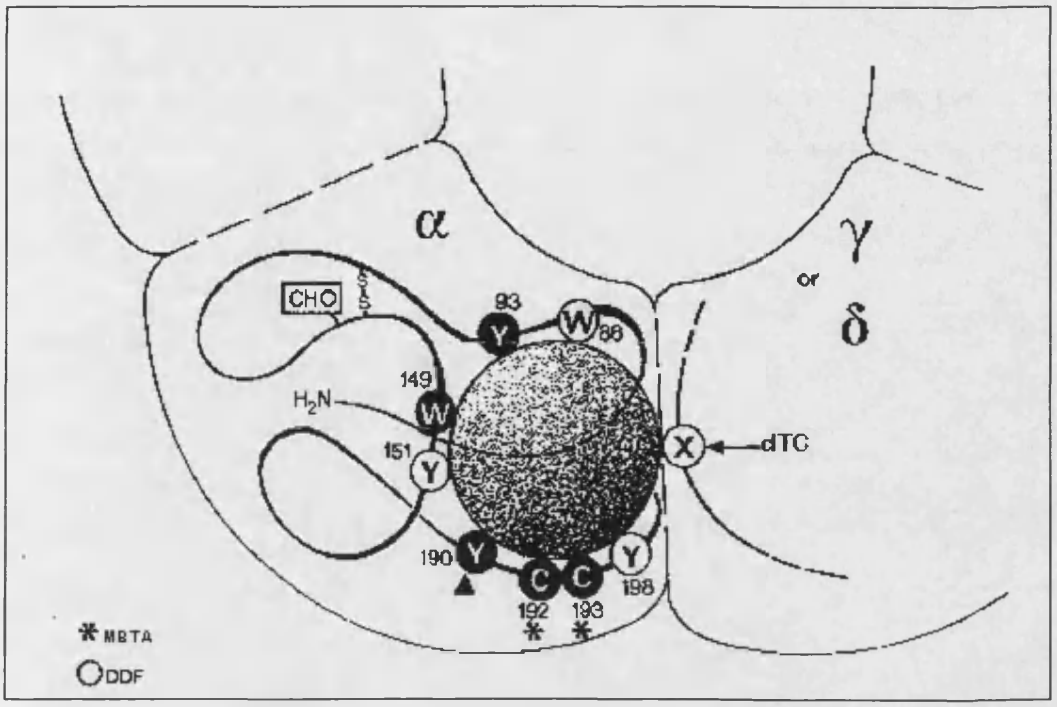
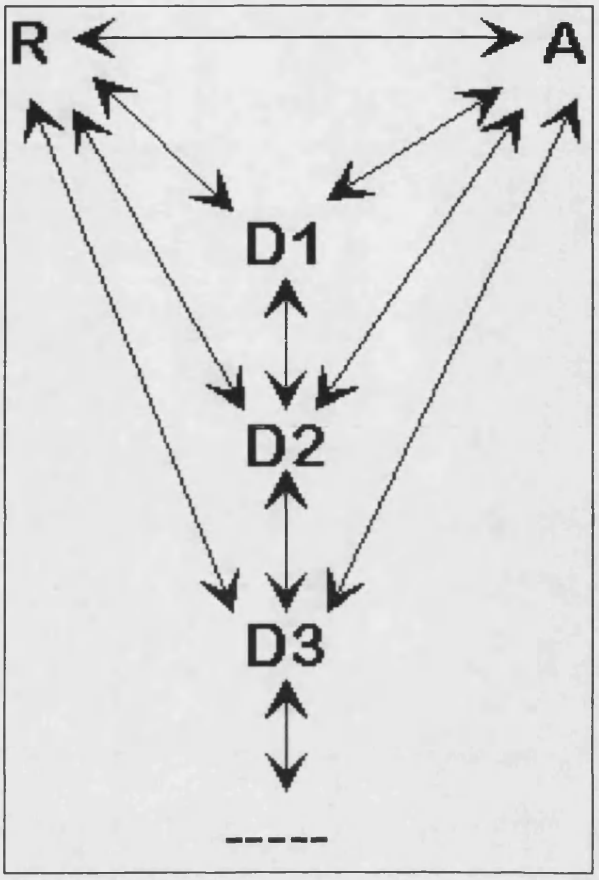


Figure 1.3

A schematic of the 'multiple loop' model of the agonist/competitive antagonist binding site of the nAChR. The large sphere represents the competitive antagonist DDF in all possible orientations. The polypeptide chain has been folded so that all labelled residues contact the sphere. The putative glycosylation site at N141 is labelled CHO. The residue labelled **X** indicates the possible involvement of residues from the γ and δ subunits in the ACh binding site (Chiara & Cohen, 1992). These residues were labelled using [^{3}H]d-tubocurarine (dTC) as an affinity ligand (modified from Galzi *et al.*, 1991a; Changeux, 1990).



R = Resting state
A = Active state
D = Desensitised state

Figure 1.4

A model of allosteric conformations of the nAChRs. This model described by Lena & Changeux (1993), was based on the 'Concerted' model of allostery proposed by Monod *et al.*, (1965). The model is explained in the text.

Subunit	Abundance	Distribution
α4, β2, β4	High	Widespread
α3	Medium	Limited: Brain stem motor nuclei, locus ceruleus thalamus, autonomic ganglia.
α6, β3	Low	Restricted: Hippocampus, thalamus autonomic ganglia.
α5, α2	Very Low	Focal: Hippocampus, cortex.

Table 1.1

The relative abundance and distribution of neuronal nAChR subunits that do not bind αBgt within the mammalian CNS (modified from Williams *et al.*, 1994).

	Agonists	Antagonists		
nAChR Subtype	**Descending Order of Potency**	**nBgt (10^{-7}M)**	**NSTX (10^{-9}M)**	**LTX-1 (10^{-5}M)**
α2β2	Nic > DMPP = ACh > Cyt	0	+++	++
α2β4	Cyt > Nic > ACh > DMPP	-	-	-
α3β2	DMPP = ACh > Nic > Cyt	+++	+++	+++
α3β4	Cyt > Nic = ACh = DMPP	0	-	-
α4β2	Nic = ACh > DMPP > Cyt	+	+++	+++
α4β4	Cyt > Nic > ACh > DMPP	-	-	-

Cyt = Cytisine
Nic = Nicotine

- **-** No Data
- **0** No Blockade of ACh Induced Current
- **+** Significant Blockade of ACh Induced Current at Toxin Concentration of 1μM
- **++** <70% Blockade of ACh Induced Current
- **+++** >70% Blockade of ACh Induced Current

Table 1.2

The pharmacological properties of neuronal nAChR subtypes expressed in *Xenopus* oocytes. For agonists, the rank order of potency was determined by *Xenopus* oocyte responses relative to the response seen with 30μM ACh. The data for the antagonists is expressed in terms of the toxins' ability to inhibit ACh induced responses in *Xenopus* oocytes.

	$\alpha 7_c$	$\alpha 8_c$	$\alpha 9_r$	$\alpha 1_c$	$\alpha 7_h$
$\alpha 7_c$	100	82	38	44	88
$\alpha 8_c$		100	38	45	70
$\alpha 9_r$			100	37	-
$\alpha 1_c$				100	38
$\alpha 7_h$					100

$_c$ = Chick
$_r$ = Rat
$_h$ = Human

- = Not Determined

Table 1.3

The sequence identity homologies of the subunits of the neuronal nAChRs that do bind αBgt.

Table 1.4

a)

* = IC_{50} of dTC not curare.
- = No data.

b)

+ = Activates channel.
- = Does not activate channel.
± = 100nM toxin transiently blocks ACh response.

ACh = Acetylcholine	Cyt = Cytisine	OXO = OXO-M
DMP = DMPP	Nic = Nicotine	Musc = Muscarine
Atrop = Atropine	Stry = Strychnine	

a) The pharmacological characteristics of homomeric and native α7 and α8 containing nAChRs.

		Agonists (EC_{50})			Antagonist (IC_{50})			
nAChR Subtype		**ACh**	**Nicotine**	**Cytisine**	**αBgt**	**Curare**	**Atropine**	**Strychnine**
Homomeric α7		$3.2x10^{-4}M$	$2.4x10^{-5}M$	$5.6x10^{-6}M$	$1.6x10^{-9}M$	$*2.7x10^{-7}M$	$3.3x10^{-4}M$	$1.5x10^{-5}M$
Native α7		$1.6x10^{-4}M$	$1.3x10^{-6}M$	$2.0x10^{-6}M$	$1.9x10^{-9}M$	$7.3x10^{-6}M$	$1.2x10^{-4}M$	$9.0x10^{-6}M$
Native α7-α8		$6.1x10^{-6}M$	$3.1x10^{-7}M$	-	$6.1x10^{-9}M$	$*2.6x10^{-6}M$	-	-
Native α8	**Site 1**	$3.1x10^{-8}M$	$1.2x10^{-8}M$	$3.5x10^{-8}M$	$2.2x10^{-8}M$	$7.9x10^{-7}M$	$5.8x10^{-7}M$	$2.0x10^{-6}M$
	Site 2	$3.9x10^{-4}M$	$1.1x10^{-5}M$	$9.4x10^{-7}M$	-	$6.5x10^{-5}M$	$5.3x10^{-5}M$	$1.8x10^{-5}M$
Homomeric α8		$1.9x10^{-6}M$	$1.0x10^{-6}M$	$1.0x10^{-6}M$	$1.4x10^{-9}M$	$*1.8x10^{-6}M$	$4.0x10^{-7}M$	$8.0x10^{-7}M$

b) The pharmacology of the homomeric α9 nAChR.

Agonists					Antagonists					
EC50					IC50					
ACh	**Nic**	**Cyt**	**OXO**	**DMP**	**Nic**	**αBgt**	**nBgt**	**Musc**	**Atrop**	**Stry**
$1.0x10^{-5}M$	-	-	+	+	$30x10^{-5}M$	±	±	$7.5x10^{-5}M$	$1.3x10^{-6}M$	$20x10^{-9}M$

Chapter 2.

2. Materials And Methods

2.1. Materials

2.1.1. Organisms

Escherichia coli TG1 [*Sup*E44 *hsd*Δ5 *thi* Δ(*lac-pro*AB) F'[*tra*D36 *pro*AB$^+$ *lacI*^{q} *lacZ*ΔM15; (Gibson, 1984)] were made competent by the $CaCl_2$ method (detailed below). *E. coli* DH5α [*Sup*E44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1; (Hanahan, 1983)] were made competent by the $RbCl_2$ method (detailed below). These strains of *E. coli* were used throughout as host strains for cloning. Cell lines COS-7 (ECACC No. 87021302) and HEK-293 (ECACC No. 85120602) were obtained from the PHLS, Porton Down, U.K.

2.1.2. Vectors

FlipCATα7Δ and the chimaeric α7-5HT$_3$ cDNA were kindly provided by M. Ballivet (University of Geneva, Switzerland) and J-L. Galzi (Institut Pasteur, France) respectively. pBluescript® II SK+ phagemid (GenBank® No. x52328) was obtained from Stratagene Ltd, Cambridge, U.K. pRc/CMV was from Invitrogen, R & D Systems Europe Ltd, Abingdon, U.K. pMT3 (Swick *et al.,* 1992) was from the Genetics Institute Inc., MA, U.S.A.

2.1.3. Enzymes

Restriction endonucleases and DNA modifying enzymes were obtained from Gibco BRL, Uxbridge, U.K. and Boehringer Mannheim, Lewes,

U.K. RNase A was from Boehringer Mannheim, Lewes, U.K. Sequenase™ sequencing kits were obtained from United States Biochemicals, Cleveland, OH, U.S.A. Biotaq DNA polymerase was obtained from Bioline U.K. Ltd, Finchley, U.K.

2.1.4. Reagents

General reagents and salts for growth media, unless otherwise specified, were from Sigma Chemicals, Poole, U.K. Yeast extract, tryptone and agar were from Difco Labs, Detroit, U.S.A. or Sigma Chemicals. Agarose was from Sigma Chemicals. Phenol was from Rathburn Chemicals Ltd, Waterburn, U.K. Oligodeoxyribonucleotide synthesis reagents were from Severn Biotech Ltd, Kidderminster, U.K. or Cruachem Ltd, Glasgow, U.K. Lipofectin™, LipofectAMINE™, fibronectin and poly-L-lysine were from Gibco-BRL. Tissue culture growth media and supplements were from Flow Laboratories, Irvine, U.K. Foetal calf serum was from SeraLab. Sequagel™ was from National Diagnostics, Atlanta, GA, U.S.A. Deoxyadenosine 5'[α-^{35}S] triphosphate and [^{125}I] were from NEN Research Products, Stevenage, U.K. Sephadex G-50, G-25 and Sepharose CL-6B were from Pharmacia, Milton Keynes, U.K. FITC-αBungarotoxin, αBungarotoxin, d-Tubocurarine Chloride Hexamethonium Bromide and Trypan Blue Solution were from Sigma.

2.1.5. Media and Solutions

The composition of media and solutions used in the Methods section are given below. Solutions were sterilised either by autoclaving at

120°C, 1.41KPa for 20 minutes, or by filtering using Millipore 0.22μM syringe filters. All solutions were stored at room temperature in colourless glass or plastic bottles, unless otherwise stated.

2.1.5.1. COS-7, HEK-293 and Bacterial Cell Growth Media

$DMEM_g$ (for general growth of COS-7 cells)

DMEM (Dulbrecco's Modified Eagle Medium)

2mM Glutamine

10U/ml Penicillin

10μg/ml Streptomycin

10% (v/v) FCS (heat inactivated)

$DMEM/F12_g$ (for general growth of HEK-293 cells)

50/50 mix of DMEM/Ham's F12 (including Glutamax I)

10U/ml Penicillin

10μg/ml Streptomycin

10% (v/v) FCS (heat inactivated)

$DMEM_t$ and $DMEM_a$ (for transfection and binding assay)

DMEM

2mM Glutamine

$DMEM/F12_t$ and $DMEM/F12_a$ (for transfection and binding assay)

50/50 mix of DMEM/Ham's F12 (including Glutamax I)

LB Broth

0.1% (w/v) Tryptone

0.5% (w/v) Yeast-Extract

0.1% (w/v) NaCl

SOB Broth

0.2% (w/v) Tryptone

0.05% (w/v) Yeast-Extract

0.005% (w/v) NaCl

2.5mM KCl

10mM $MgCl_2$

LB Agar

LB Broth

1.5% (w/v) Agar

2.1.5.2. Competent Cell Solutions

RFI

100mM RbCl

50mM $MnCl_2$

30mM Potassium Acetate

10mM $CaCl_2$

15% (v/v) Glycerol

pH5.8 (Acetic Acid)

RFII

10mM MOPS

10mM RbCl

75mM $CaCl_2$

15% (v/v) Glycerol

pH6.8 (KOH)

Both solutions were then filter sterilised

2.1.5.3. Agarose Gel Electrophoresis Solutions

0.5x TBE Buffer

44.5mM Tris\Orthoboric Acid (pH8.0)

1.25mM EDTA

10x Loading Buffer

50% (v/v) Gluteraldehyde

0.25% (w/v) Bromophenol Blue

0.25% (w/v) Xylene Cyanol

2.1.5.4. DNA Isolation Solutions

GTE Buffer

25mM Tris\HCl (pH 8.0)

10mM EDTA

50mM Glucose

Standard Lysis Buffer

200mM NaOH

1% (w/v) SDS

Made up just prior to use.

Rapid Lysis Buffer

50mM Tris/HCl (pH8.0)

2.5M LiCl

4% (v/v) Triton X-100

62.5mM EDTA

TE Buffer

10mM Tris/HCl (pH8.0)

1mM EDTA

2.1.5.5. Iodination Solutions

Reaction Buffer

50mM KH_2/K_2HPO_4

pH 7.5

Column Elution Buffer

1:4 Dilution of Reaction Buffer (with distilled water)

1% (w/v) BSA

Chloramine-T Solution

5% (w/v) Chloramine T (in Reaction Buffer)

Made up just prior to use.

Sodium Metabisulphite

0.08% (w/v) $Na_2S_2O_5$ (in Reaction Buffer)

Made up 1:4 Dilution in Reaction Buffer just prior to use.

KI

1% (w/v) KI (in Reaction Buffer)

Made up just prior to use.

2.1.5.6. Protein Determination Solutions

Lowry Solution A

2% (w/v) Na_2CO_3

0.4% (w/v) NaOH

0.16% (w/v) KNaTartrate

1% (w/v) SDS

Lowry Solution B

4% (w/v) $CuSO_4.5H_2O$

2.1.5.7. Fluorescence Assay Solutions

0.2M Phosphate Buffer

0.8% (w/v) KH_2PO_4

2% (w/v) Na_2HPO_4

pH7.2

Formaldehyde Fixing Solution

48ml Hot Distilled H_2O (60°C)

4g Paraformaldehyde

10-12 drops 1M NaOH

50ml 0.2M Phosphate Buffer

The solution was filtered through Whatman No.1 filter paper.

2.1.5.8. General Solutions

Chloroform	24:1 (v/v) Chloroform:isoamylalcohol
Phenol	Obtained distilled and stored at 4°C, equilibrated with 2vols. 50mM Tris/HCl pH8.0. Stored in the dark at -20°C.
Phenol/Chloroform	Equal volumes of equilibrated Phenol and Chloroform/isoamylalcohol. Stored in the dark at -20°C
Ethidium Bromide	10mg/ml stock, stored in the dark at 4°C
RNase A (DNase Free)	10mg/ml stock in TE Buffer, incubated at 95°C for 30 minutes and stored at -20°C

PBS

0.8% (w/v) NaCl

0.02% (w/v) KCl

0.144% (w/v) Na_2HPO_4

0.024% (w/v) KH_2PO_4

2.1.6. Equipment

All water used was from the Milli-Q™ reagent grade water system from Millipore Corp., Bedford, MA, U.S.A. Oligodeoxyribonucleotides were synthesised on an Applied Biosystems 381A DNA Synthesiser. The DNA amplification was carried out in a PHC-2 from Techne, Cambridge, U.K. or a DNA Thermal Cycler™ from Perkin-Elmer-Cetus, Norwalk, CT, U.S.A. Centrifugation was carried out in an Ole Dich microfuge 154 or a Du Pont Instruments RCB5 centrifuge or a Beckmann L5-65 ultracentrifuge. X-ray autoradiography film was from Fuji Photo Films Co., London U.K. Film cassettes, film processing chemicals, Polaroid film and equipment were supplied by Eastman Kodak Co., Liverpool, UK. Tissue culture vessels were from Nunc/Gibco-BRL, Paisley, U.K. DNA and amino acid sequence information was compiled, stored and analysed using the Staden-Plus™ package from Amersham International Plc., Aylesbury, U.K. and the 'gnome' database at the University of Bath, Bath, U.K. [^{125}I]-αbungarotoxin was counted in a Cobra™ II from Canberra-Packard, Pangbourne, U.K. Statistics and graphs were generated using Microsoft Excel© (Ver. 5.0a) from Microsoft Corporation, U.S.A., Biosoft Fig.P© (Ver. 6.2) from Fig.P Software Corporation, U.S.A. and Sigma-Plot from Jandel Scientific GmbH, Germany.

2.2. Methods

2.2.1. Preparation of Competent Cells

2.2.1.1. $CaCl_2$ Method

Competent *E. coli* TG1 cells were prepared using the $CaCl_2$ method described by Sambrook *et al.,* (1989). A 10µl aliquot from a glycerol stock of *E. coli* TG1 was used to inoculate 5ml LB broth and grown overnight. An 100µl aliquot of this overnight culture was used to inoculate 100ml LB broth and grown at 37°C until the OD_{600} reached 0.4. The cells were then centrifuged at 10,000xg for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 20ml ice cold 50mM $CaCl_2$, then incubated on "wet" ice for 30 minutes. The cells were pelleted as before and resuspended in 2ml ice cold 50mM $CaCl_2$. The cells were ready for transformation after 1 hour and up to 15 hours thereafter.

2.2.1.2. $RbCl_2$ Method

Competent *E. coli* DH5α cells were prepared using a modified $RbCl_2$ method described by Perbal (1988). A 10µl aliquot from a glycerol stock of *E. coli* DH5α was taken and grown overnight in 5ml SOB broth. An 100µl aliquot of this overnight culture was used to inoculate 50ml SOB broth and grown until the OD_{600} reached 0.4. The cells were centrifuged in pre-cooled tubes at 1,000xg for 10 minutes at 4°C. The pelleted cells were resuspended in 40ml ice cold RFI and incubated on ice for 5 minutes. Then the cells were pelleted as before. The pellet was resuspended in 8ml ice cold RFII and incubated overnight on ice. The competent cells were divided into

100µl aliquots then flash frozen in liquid N_2. The competent cells were stored at -70°C until required.

2.2.2. Transformation of Competent Cells

2.2.2.1. Transformation of $CaCl_2$ Competent *E. coli* TG1

Competent *E. coli* TG1 cells were transformed with plasmid DNA or recombinant plasmid DNA using a method based on that described in Sambrook *et al.,* (1989). The DNA sample was mixed with 300µl competent cells and kept on ice for 30 minutes. The cells were 'heat-shocked' in a 42°C water bath for 3 minutes. Then 1ml prewarmed LB broth was added to the cells and they were incubated in a 37°C water bath for 45 minutes. The cells were then plated out on LB agar containing ampicillin (100µg/ml) and incubated at 37°C for 12-18 hours.

2.2.2.2. Transformation of $RbCl_2$ Competent *E. coli* DH5α

$RbCl_2$ competent cells were thawed on ice for 15 minutes. The sample DNA was added to 50µl cells in pre-cooled eppendorf tubes and. The cells were incubated on ice for 30 minutes, "heat-shocked" at 42°C for 90 seconds, then returned to ice for 1 minute. Three volumes of SOB broth were added to the cells and then they were incubated for 1 hour at 37°C in a shaking incubator. The cells were then plated out on LB agar containing ampicillin (100µg/ml) and incubated at 37°C for 12-18 hours.

2.2.3. Small-Scale Preparation of Plasmids

2.2.3.1. Standard Alkaline Lysis Method

This method is based on that described by Sambrook *et al.*, (1989). Transformants of interest were grown overnight in 5ml LB broth containing ampicillin (100μg/ml). An 1.5ml sample of culture was centrifuged at 20,000xg for 5 minutes, the rest of the culture was stored at 4°C. The supernatant was discarded and the pellet resuspended thoroughly in 100μl of GTE. Then 200μl of Standard Lysis Buffer was added and mixed thoroughly. Then 150μl of 5M Potassium acetate (pH4.6) was added and mixed thoroughly. The white precipitate was centrifuged at 20,000xg for 5 minutes and the supernatant decanted and kept. The supernatant was then solvent extracted with phenol/chloroform (1:1). The aqueous phase was transferred to a fresh tube. The nucleic acids were precipitated from the aqueous phase with 1ml absolute ethanol. The pellet was washed in 500μl 70% (v/v) ethanol and vacuum dried. The pellet was resuspended in 50μl of sterile distilled water and 1μl RNase A was added. This DNA was then used in restriction endonuclease digestions.

2.2.3.2. Rapid Method

This method is based on that described be He *et al.*, (1990). Transformants of interest were grown overnight in 5ml LB broth containing ampicillin (100μg/ml). An 1.5ml sample of culture was centrifuged at 20,000xg for 30 seconds, the rest of the culture was stored at 4°C. The supernatant was carefully aspirated away and the pellet resuspended/lysed in 100μl

Rapid Lysis Buffer. Then 100µl phenol/chloroform (1:1) was added to the lysate and this mix was vortexed for 15 seconds. The phases were separated by centrifuging at 20,000xg for 1 minute at room temperature. The aqueous phase was transferred to a fresh tube, 200µl of absolute ethanol was added to this phase and mixed by inverting the tube. The tube was then centrifuged at 20,000xg for 10 minutes at 4°C. The supernatant was carefully aspirated away. The pellet was washed with 1ml of 70%(v/v) ethanol, centrifuged for 1 minute as before and the supernatant aspirated as before. The pellet was vacuum dried for 5 minutes and redissolved in 30µl TE Buffer. This DNA was then used in restriction endonuclease digestions.

2.2.3.3. Promega Wizard™ Minipreps and Wizard™ Clean-Up

These commercially available kits use an affinity resin to isolate the DNA from the cell lysate or PCR reaction. The manufacturer's instructions were followed using solutions provided with the kits.

2.2.4. Large-Scale Preparation of Plasmids

2.2.4.1. Alkaline Lysis Method Using CsCl and EtBr

This method is also based on the alkaline lysis protocol that is described in Sambrook *et al.,* (1989). From the cells stored at 4°C, 500µl was used to inoculate 500ml LB broth with ampicillin (100µg/ml). The cells were grown overnight, then pelleted by centrifugation at 3,000xg for 20 minutes at 4°C. The pellet was resuspended in 8ml of GTE. The cells were lysed by adding 16ml Standard Lysis Buffer and then incubated on ice for 10 minutes.

The cell debris was precipitated with 5ml 5M Potassium acetate (pH4.6) and then pelleted by centrifuging at 10,000xg for 30 minutes at 4°C. The supernatant was decanted and kept. Nucleic acids were precipitated from the supernatant with 0.6vols. of propan-2-ol at room temperature, then pelleted by centrifugation at 10,000xg for 10 minutes at 4°C. The vacuum dried pellet was resuspended in 10ml of sterile distilled water and the volume accurately noted. An equal weight (in g) of CsCl was added, with 300μl of 10mg/ml Ethidium Bromide (EtBr) and this solution transferred to Beckmann heat sealable tubes. The tubes were balanced to within 10mg, sealed and centrifuged at 340,000xg for 18 hours.

The lower, supercoiled plasmid band was taken and solvent extracted with amylalcohol to remove the EtBr. The aqueous phase was diluted with 1ml sterile distilled water and then the DNA precipitated with 3vols. ice cold absolute ethanol. The vacuum dried pellet was resuspended in 1ml sterile distilled water, then precipitated with 2ml absolute ethanol plus 100μl 3M Sodium acetate. The vacuum dried pellet was resuspended in 1ml sterile distilled water and precipitated as before. This pellet was resuspended in 200μl sterile distilled water.

2.2.4.2. Alkaline Lysis Method Using LiCl

From the cells at 4°C, 100ml LB broth was inoculated, cells pelleted and resuspended in 4ml GTE as before. The cells were lysed with 8ml Standard Lysis Buffer and incubated on ice for 10 minutes. Cell debris from the lysate was precipitated with 15ml ice cold 5M Potassium acetate

(pH4.6) and the suspension incubated on ice for 15-30 minutes. The suspension was centrifuged at 1,000xg for 15 minutes at room temperature. The supernatant was filtered through muslin into another tube. Nucleic acids were precipitated by adding 18ml propan-2-ol and incubating on ice for 30 minutes. The nucleic acids were pelleted by centrifugation for 30 minutes as before and the air dried pellet resuspended in 1ml TE Buffer. The suspension was split between two tubes, 500µl 6M LiCl added to each tube and incubated on ice for 15 minutes. The RNA/protein/salt complex was pelleted at 20,000xg for 10 minutes at room temperature. The supernatants were split between two tubes and 1ml of -20°C absolute ethanol added to each tube and incubated at -20°C for 30 minutes. The nucleic acids were pelleted by centrifuging for 10 minutes at 20,000xg at room temperature. The pellets were then washed in 500µl 70% (v/v) ethanol and repelleted by centrifugation as before. The pellets were vacuum dried and collated by resuspension in 400µl TE Buffer. RNA was removed by the addition of 4µl RNase A and incubation at 37°C for 30 minutes. The sample was further incubated with 20µl SDS (10% (w/v)) at 75°C for 10 minutes. Then 420µl 6M LiCl was added and incubated for 15 minutes at room temperature before centrifugation as before. The supernatant was split between two tubes, 1ml absolute ethanol was added to each tube and the tubes incubated on ice for 30 minutes. The DNA was pelleted by centrifugation at 20,000xg for 30 minutes at room temperature. The pellets were washed in 500µl 70% (v/v) ethanol, then repelleted by centrifugation for 10 minutes at 20,000xg at room temperature. The pellets were resuspended in 400µl sterile distilled H_2O, then solvent

extracted with an equal volume of phenol/chloroform (1:1). This was followed by 2 further equal volume chloroform extractions, then an absolute ethanol precipitation. The pelleted DNA was resuspended in 100μl sterile distilled H_2O and could be used in further procedures.

2.2.5. Determination of Plasmid DNA Concentration, Purity and Oligodeoxyribonucleotide Concentration

DNA concentration was determined in aqueous solution spectrophotometrically by measurement of absorbency at 260nm (A_{260}), using 1 absorbance unit = 50μg double stranded DNA per ml. DNA purity was assessed spectrophotometrically by measurement of the ratio of absorbance A_{260}/A_{280}. A value ≅ 1.8 for double stranded DNA was used as a criteria for purity.

Oligodeoxyribonucleotide concentration was determined spectrophotometrically by measurement of A_{260}, using 1 absorbance unit = 33μg single stranded DNA per ml. This value was converted to molarity using the equation below.

$$\frac{[Sample]}{325 \times l}$$

[Sample] Spectrophotometrically determined concentration of sample in grammes per litre

l Length of oligodeoxyribonucleotide

325 Average molecular weight of a nucleotide

2.2.6. Sequencing

The template DNA (10μg double stranded) was denatured in 15μl Denaturing buffer (1M NaOH\1mM EDTA) at room temperature for 5 minutes. The denatured DNA was then put down a 400μl Sepharose CL-6B column and the eluate stored on ice until required. For each Sequenase™ T7 polymerase reaction 7μl of the column eluate was used. The kit used the "Dideoxyribonucleotide Chain Termination" method based on those described by Sanger *et al.,* (1977) and Messing, (1983). The manufacturer's instructions were followed using the solutions provided in the kit and [α-^{35}S]-dATP. Then the sequences were separated by electrophoresis in TBE through denaturing 6% (w/v) acrylamide/urea Sequagel™ gels, at 30mA constant current. Gels were fixed and the urea eluted by washing for 30 minutes in 5% (v/v) Acetic acid/5% (v/v) Methanol in distilled water. The gels were washed in distilled water, dried and placed, still attached to the glass plate, in an autoradiography cassette with X-ray film. The film was exposed to the gel for at least 16 hours prior to development.

2.2.7. Agarose Gel Electrophoresis

Agarose was dissolved in very hot 0.5x TBE and allowed to cool to 50°C. EtBr was added to a final concentration of 500μg/l and the solution used to pour horizontal slab gels. In general 1% (w/v) agarose gels were poured. Low melting point agarose gels (1% (w/v)) were poured when DNA fragments were to be isolated. The DNA samples were separated by electrophoresis in 0.5x TBE containing 100μg/l EtBr. The gels were run at

between 100-150 mA constant current, with *Hin*DIII or *Pst*I digested λ-phage DNA as molecular weight markers.

2.2.8. Isolation of DNA Fragments From Agarose Gels

A simple gel filtration method using Sephadex G-50 method was employed to recover desired DNA fragments from agarose gels. This method was as described by Mukhopadhyay and Roth, (1991).

2.2.9. Preparation of Oligodeoxyribonucleotides for Use in PCR and Sequencing

Specific biotinylated oligodeoxyribonucleotides were synthesised using β-cyanoethyl phosphoramidite chemistry on an Applied Biosystems DNA synthesiser according to the manufacturer's instructions. The oligodeoxyribonucleotides were deprotected and eluted from the solid support by incubation with 1ml NH_3 solution (d = 0.89g/ml) for 6-18 hours at 55°C. The NH_3 solution was neutralised by the addition of 1ml Acetic acid, after incubation at -20°C for 5 minutes. The oligodeoxyribonucleotides were precipitated by the addition of 6ml -20°C absolute ethanol and incubation at -20°C for 30 minutes. The oligodeoxyribonucleotides were then pelleted by centrifugation at 400xg for 5 minutes at room temperature. The supernatant was discarded and the pellet washed in 3ml 80% (v/v) ethanol and centrifuged as before. The pellet was vacuum dried and resuspended in 1ml sterile distilled H_2O. This suspension was clarified by centrifuging if

necessary, then the concentration and purity determined prior to storage at -20°C.

2.2.10. Polymerase Chain Reaction

The ability to amplify, clone and ultimately sequence trace amounts of DNA is provided by PCR (Saiki *et al.,* 1985). This technique involves repeated cycles of high temperature denaturation of double stranded DNA, followed by primer annealing to the separated strands of DNA and primer extension via the use of dNTPs and a DNA polymerase. Since the DNA denaturation usually involves temperatures of ≅95°C, a thermostable DNA polymerase must be used. The most common is *Taq* DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus*. *Taq* DNA polymerase retains its activity after >30 minutes at 94°C. Using conserved or mismatched primers, genes/cDNA can be isolated or site directed mutagenesis can be performed using PCR.

The PCR reactions were set up as follows -:

1ng	Target DNA
1.5-2μM	Oligomer Primers
1.25mM	dNTPs (dATP, dTTP, dGTP and dCTP)
10μl	10x *Taq* Polymerase Buffer

Made up to 99.5μl with sterile distilled water.

0.5μl	Taq Polymerase (5U/ml)

The PCR reaction was overlaid with a layer of mineral oil to prevent evaporation from the reaction tube. Using 95°C for 60 seconds to denature the double stranded DNA, 48°C for 60 seconds to anneal the primers and 72°C for 90 seconds to extend the primers, the PCR reaction was cycled 30 times.

2.2.11. Dynal Streptavidin-Coated Magnetic DynaBeads

Dynabeads (20μl) were taken, stuck to the magnet and the supernatant removed. The beads were then washed in 50μl Binding Buffer (10mM Tris/HCl pH7.5 1mM EDTA 2M NaCl). The beads were stuck to the magnet and the supernatant removed, this procedure was repeated. A PCR reaction using biotinylated primers, was affinity purified using a Promega Wizard™ Clean-up kit. To the eluate from the Promega Wizard™ Clean-up kit, 100μl Binding Buffer was added. The streptavidin/biotin interaction was allowed to continue for 13-18 hours at 37°C, with agitation. The beads were washed twice in 100μl sterile distilled H_2O and the first restriction endonuclease digestion set up. The digestions were performed at 37°C overnight. The beads were washed in sterile distilled H_2O as before and the second digestion set up. The beads were stuck to the magnet and the supernatant, containing the suitably digested fragment, was collected and used in the next cloning step.

2.2.12. Tissue Culture

2.2.12.1. Growing of COS-7 and HEK-293 Cells

COS-7 cells were grown using DMEM as the basic media component. HEK-293 cells were grown using a 50/50 mix of DMEM and Ham's F12 as the basic media component.

2.2.12.1.1. Growing COS-7 Cells From Liquid N_2 Stocks

Prewarmed $DMEM_g$ (5ml) was placed in a 50ml culture flask along with 1ml of defrosted stock COS-7 cells. The cells were then placed in a humidified incubator at 37°C, with a 5% (v/v) CO_2 atmosphere. After allowing two hours for cell attachment to culture vessel surface, the media was replaced with another 5ml $DMEM_g$. This was to remove the DMSO in the freezing medium that would retard normal cell growth. The $DMEM_g$ was changed every 72-96 hours.

2.2.12.1.2. Growing HEK-293 Cells From Liquid N_2 Stocks

A 1ml liquid N_2 stock of HEK-293 cells was defrosted quickly at 37°C and the phial topped up with prewarmed $DMEM/F12_g$. The cells were pelleted by centrifuging the phial at 70-80xg for 5 minutes at room temperature. The supernatant was carefully aspirated off and discarded, while the pellet was resuspended gently in 1ml of prewarmed $DMEM/F12_g$. The cell suspension was then added to a 50ml culture flask, containing 5ml of prewarmed $DMEM/F12_g$. The cells were then placed in a humidified incubator at 37°C, with a 5% (v/v) CO_2 atmosphere for 48 hours, to facilitate cell

attachment to the surface of the culture flask. The DMEM/F12$_g$ was changed every 72-96 hours.

2.2.12.2. Passaging of COS-7 and HEK-293 Cells

When the cells were 95-100% confluent the cells were passaged into 250ml flasks. The media was poured off the cells and the traces of media removed by washing monolayer with prewarmed PBS. The cells were then trypsinised (0.025% (w/v) Trypsin solution, in prewarmed PBS) for 5 minutes at 37°C and the trypsinisation halted by the addition of 10ml of prewarmed DMEM$_g$ or DMEM/F12$_g$ The cell suspension was then centrifuged at 200xg for 5 minutes at room temperature. The supernatant was discarded and the pellet gently resuspended in 5ml prewarmed DMEM$_g$ or DMEM/F12$_g$ 1ml of this suspension was then used to seed 20ml of prewarmed DMEM$_g$ or DMEM/F12$_g$ in a 250ml flask.

2.2.12.3. Transfection of COS-7 and HEK-293 Cells

Transfection was based on the cationic liposome-mediated method described by Felgner *et al.,* (1987) using DOTMA (*N*[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride) or DOSPA (*N*-[2-({2,5-bis[(3-aminopropyl)amino]-1-oxypentyl}amino)ethyl]-*N,N*-dimethyl-2,3-bis (9-octadecenyloxy)-1-propananium trifluoroacetate) as the cationic lipid agents. DOTMA and DOSPA were supplied as Lipofectin™ and LipofectAMINE™ reagents, respectively. The cells were plated out into 100mm culture dishes and when the cells were between 30-50% confluent, the manufacturers

instructions were followed for transfection. A transfection reagent/DNA complex of 4μl/2μg per 9.6cm^2 was used. After a 6 hour incubation with $DMEM_t$ or $DMEM/F12_t$, transfection was halted by the addition of prewarmed $DMEM_g$ or $DMEM/F12_g$ supplemented with 20% FCS. Relevant toxins and channel blockers were added to the cells at this point. Also at this point, percentage cell viability was determined using the 'trypan blue exclusion' method. The next day the cells were trypsinised as before into fibronectin/poly-L-lysine coated 35mm, 6 well plates or onto fibronectin/poly-L-lysine coated cover slips. Approximately 24 hours after trypsinising, the [^{125}I]-αBgt and FITC-αBgt binding assays were performed on the cells.

2.2.12.4. Trypan Blue Exclusion Viable Cell Assay.

Cells to be counted were pelleted by centrifugation at 200xg for 5 minutes at room temperature. The pellet was resuspended in 1ml PBS, 200μl of this cell suspension was added to a tube containing 500μl Trypan Blue Solution and 300μl PBS. This suspension was mixed thoroughly and left to stand for 5 minutes at room temperature. A sample of this suspension was used to fill an hemocytometer by capillary action. Viable cells (ie. live cells) are not stained by the dye and appear translucent. Non-viable cells (ie. dead cells) are stained by the dye and appear black/dark blue in colour. The numbers of viable and non-viable cells were counted and noted in 5 squares of the hemocytometer, excluding the cells in contact with the upper and right borders of the squares. This procedure was repeated on a second aliquot of

the Trypan Blue/cell suspension. Calculations for total % cell viability were then carried out.

$$\text{Total \% Cell Viability} = \frac{\sum \text{Viable Cells(Unstained)}}{\sum \text{Cells(Stained \& Unstained)}} \times 100\%$$

2.2.13. [^{125}I] Procedures

2.2.13.1. Iodination of αBgt

Iodination of αBgt was based on the Chloramine-T method described by Urbaniak *et al.,* (1973). Reaction Buffer (10μl), 20μl αBgt (125μM), and 20μl [^{125}I] (0.1mCi/μl) were placed in a tube and the reaction started by the addition of 10μl Chloramine-T Solution. After 1 minute at room temperature the reaction was stopped by the addition of 750μl $Na_2S_2O_5$ and 200μl KI. The toxin incorporated [^{125}I] and free [^{125}I] were separated on a 25ml Sephadex G-25 column, in Column Elution Buffer. Twenty 1ml fractions were collected from the column and 5μl of each fraction countered in a γ-counter. From these results specific activity and % incorporation were determined.

2.2.13.2. [^{125}I]-αBgt Binding Assay

This assay was based on that described by Gu *et al.,* (1990) and Chavez *et al.,* (1992). The growth media was poured off and the traces of media removed completely by washing cells three times in 1ml prewarmed PBS. Surface expression of toxin binding proteins was determined by incubation of cells with 10nM [^{125}I]-αBgt in 500μl $DMEM_a$ or $DMEM/F12_a$ for 1

hour at 37°C. Non-specific binding was determined by the inclusion of 1μM "cold" αBgt. Cells were then washed twice in 1ml PBS, then removed from the dish surface with 1ml Lowry Solution A. The samples were then counted in a γ-counter and afterwards used in a protein determination assay.

2.2.14. Protein Determination Assay

This assay was based on the modified Lowry protein determination assay described by Markwell *et al.,* (1978). BSA (1mg/1ml, in Lowry Solution A) was used to generate a standard curve from 0-200μg of protein. One hundred parts Lowry Solution A was mixed with one part Lowry Solution B and 1ml aliquots added to 200μl of sample. The solutions were mixed and incubated at room temperature for 10 minutes. Folin-Ciocalteu reagent was diluted 1:1 with distilled H_2O and 100μl of the diluted reagent added to each sample. The solutions were mixed and incubated at room temperature for 45 minutes, before determining the A_{750}. Linear regression of the BSA standard curve was carried out and the protein concentrations of the samples determined by the equations generated.

2.2.15. Fluorescence Binding Assay

The cells grown on cover slips were washed twice in 1ml PBS to remove all traces of media. The cells were then fixed for 30 minutes at room temperature in 1ml Formaldehyde Fixing Solution. The cells were washed 3 times in 1ml PBS. Total binding was determined by incubating cells with 100nM FITC-αBgt in PBS. Non-specific binding was determined by

incubating cells with FITC-αBgt/1μM αBgt in PBS. The incubation lasted for 30-40 minutes in an humidified incubator at 37°C, with a 5% (v/v) CO_2 atmosphere. The cells were then washed 4 times with 1ml PBS, mounted with glycerol or Vectashield (Vector Labs) and visualised using an inverted Nikon 'Diaphat' microscope under epifluorescent illumination and phase contrast.

2.2.16. Electrophysiology

The homomeric channels were expressed in *Xenopus laevis* oocytes. The oocytes were prepared, injected and voltage clamp recordings performed as described in Bertrand *et al.,* (1991). This work was kindly done by M. Amar and S. Wheeler.

Chapter 3.

3. Characterisation of the Homomeric Chick α7 nAChR Expressed in Mammalian Cell Lines

3.1. Introduction

This chapter will introduce the cell lines, vectors and antagonists used throughout this work. Then the results of the transfection experiments, with reference to the binding assays, will be presented and discussed.

3.1.1. The COS-7 Cell Line

The COS-7 cell line was derived from the CV-1 Green monkey kidney fibroblast cell line (Gluzmen, 1981), which is permissive for the lytic growth of SV40 (Simian Virus 40). SV40 belongs to the *Papovaviridae* family of small DNA genome viruses. There are three genera in the family; *Papilloma*, *Polyoma* and *Vacuolating*. SV40 was believed to belong to the *Vacuolating* genus, but has now been classified in the *Polyoma* genus. CV-1 cells were infected with an *ori*-defective (origin-defective) mutant of SV40, that coded for the 'Large T' antigen. Hence, the cell line name COS from CV-1, Origin, SV40.

The M_r 90,000 'Large T' antigen is one of SV40's 'Early' gene products and has a possible role as a transcription factor (Gluzman *et al.*, 1980). Mutant SV40 deficient in 'Large T' antigen cannot, in permissive cells, initiate viral DNA synthesis for productive infection, late RNA synthesis or

neoplastic transformation (Fraenkal-Conrat & Kimball, 1982). COS-7 cells, transfected with a vector bearing a functional SV40 *ori*, would treat the vector as an infecting virus. Appropriately introduced cDNA sequences within the vector would be transcribed and translated. Since it is a eukaryotic expression system most, if not all the post-translational modifications and membrane direction procedures should be performed correctly.

3.1.2. The HEK-293 Cell Line

HEK stands for Human Embryo Kidney (ie. the epithelial cell source from which the cell line was derived). The HEK cells were transformed by exposure to mechanically sheared fragments of human Ad5 (Adenovirus type 5) DNA (Graham *et al.,* 1977). The DNA was sheared because, as with SV40 and CV-1 cells, HEK cells are permissive for the lytic growth of Ad5. Human Ad5 is a member of the *Adenoviridae* family. There are 2 genera in the family Aviadenovirus and Mastadenovirus, to which Ad5 belongs. (Hull *et al.,* 1989). HEK-293 cells produce many 'Early' gene products, some of which act on the AdMLP (Adenovirus Major Late Promoter) (Ginsberg, 1984). HEK-293 cells should treat as an infecting Adenovirus, vectors containing the AdMLP. Consequently cDNA sequences introduced appropriately in front of the AdMLP would be transcribed, translated and post-translationally modified.

3.1.3. Vectors

The $\alpha 7$ cDNA was subcloned from the Flip vector into the pBluescript II SK+ vector using *Hin*DIII/*Bam*HI sites (Figure 3.1). The

pBluescript II SK+ was used as an transition vector where the α7 cDNA could be manipulated and sequenced.

For COS-7 cell expression, the α7 cDNA was subcloned from pBluescript II SK+ into the pRc/CMV vector using *Hin*DIII/*Xba*I (Figures 3.1 & 3.2a). The pRc/CMV vector uses a human CMV (Cytomegalovirus) 'Immediate Early' promoter. CMV is a member of the *Betavirusherpesviridae*, a subfamily of the *Herpesviridae*. Along with its upstream enhancer sequences, the 'Immediate Early' promoter is unusually strong and competes efficiently for RNA polymerase II (Stinski, 1990). The vector also possesses the SV40 *ori* for maintenance within the COS-7 cell line.

Figure 3.2b shows the Swick *et al.,* (1992) pMT3 vector used in the HEK-293 expression system. The α7 cDNA was subcloned from pBluescript II SK+ into the pMT3 vector using *Pst*I/*Not*I (Figure 3.1 & 3.2b). pMT3 has an AdMLP, with flanking SV40 enhancer and tripartite leader sequences to increase the efficiency of RNA transcription (Kaufman, 1985). Both vectors can be used in *Xenopus* oocyte expression systems (Amar *et al.,* 1993; Swick *et al.,* 1992), enabling comparison between cultured cell- and *Xenopus* oocyte-expressed homomeric α7 nAChRs to be made.

3.2. The Antagonists

Four antagonists were used during this work. αBgt has been already mentioned. The remaining three are Hexamethonium, *d*-Tubocurarine (dTC) and Methyllycaconitine (MLA).

When NMDAR1/NMDAR2A glutamate receptors were expressed in cultured cells, cell death resulted in all the transfected cells (Cik *et al.,* 1993). This cell mortality was believed to be due to the L-glutamate present in the cell culture medium. The L-glutamate activated the expressed NMDA receptors permitting unregulated Ca^{2+} to enter the cells, eventually leading to cell death. (Cik *et al.,* 1993; Tymianski *et al.,* 1993a, 1993b). This problem was overcome by the inclusion of NMDA glutamate receptor antagonists, AP5 (DL-2-amino-phosphonopentanoic acid) and 5,7-dichlorokynurenic acid in the post-transfection medium (Cik *et al.,* 1994; Rothman & Olney, 1987). This resulted in a 25% increase in cell viability. Specific binding of [^{3}H]-MK801 also increased from ≅100 binding sites/mg protein to 976 binding sites/mg protein.

The α7 containing nAChRs (especially the homomeric α7 nAChR) have been shown to be very permeable to Ca^{2+}, even more so than the NMDA glutamate receptors. Hexamethonium and dTC (Figure 3.3), nicotinic antagonists, were included in the post-transfection medium to evaluate their effect on the expression of α7 nAChR. MLA has been recently isolated and characterised as one of the most potent low M_r molecules to discriminate between nj/e nAChRs and αBgt binding neuronal nAChRs (Wonnacott *et al.,* 1993; Sattelle *et al.,* 1989).

3.2.1. Hexamethonium

Hexamethonium, its full name N,N,N,N',N',N'-hexamethyl-1,6-hexanediaminium, was developed in 1948 as an ganglionic blocking and

antihypertensive agent (Burger, 1980). Since 1949, hexamethonium, along with the related decamethonium (used as a skeletal muscle relaxant in anaesthesia; Paton & Zaimis, 1949), has been used to distinguish between neuronal and nj/e nAChRs. Hexamethonium (C6) was better at blocking autonomic ganglia than decamethonium (C10), hence neuronal nAChRs became known as 'C6' receptors (Sargent, 1993; Paton & Zaimis, 1949). The reverse was true at the nj/e nAChRs and these receptors became known as 'C10' receptors. Ascher *et al.,* (1978) showed that hexamethonium worked by reversibly blocking the activated channel. This indicated that it was a non-competitive antagonist.

3.2.2. dTC

dTC was isolated from the tuber and leaves of *Chrondrodedron tomentosum* (King, 1935) and was used initially, like decamethonium, in anaesthesia as a skeletal muscle relaxant (King, 1946). dTC seems to inhibit the function of AChRs in two fashions.

1) As a competitive antagonist: dTC competed for the same binding site as ACh and other competitive nicotinic ligands, such as αBgt and carbamylcholine (Pedersen & Cohen, 1990; Blount & Merlie, 1989; Neubig & Cohen, 1979).

2) As a non-competitive antagonist: dTC reversibly blockaded nAChRs in a voltage dependent manner, similar to the blockade produced by histrionicotoxin, a non-competitive nicotinic antagonist (Sattelle *et al.,* 1989; David & Sattelle, 1984). Phencyclidine and proadifen, other nicotinic non-

competitive antagonists, reduce the incorporation of [^{3}H]-dTC into nAChRs in the presence of carbamylcholine (Pedersen & Cohen, 1990).

The above information indicates that as well as acting as a competitive antagonist, dTC acts as a channel blocker (Shaker *et al.,* 1982; Katz & Miledi, 1978).

3.2.3. MLA - Methyllycaconitine

Historically, the seeds' extracts of *Delphinium*, *Aconitum* and *Consolida* have been used as sources of poisons and medicines (Blagbrough *et al.,* 1994a). Today, these plants are held responsible for more cattle deaths in North America than any other poisonous plant and several 'herbal medicine' poisonings of humans (Coates *et al.,* 1994a; Tomlinson *et al.,* 1993). Pliny the Elder (G. Plinius) in AD77 documented how the topical application of pounded 'Staphis' (*D. staphysagria*) "rid the head of lice as well as the rest of the body". MLA (also known as delatine, delsemidine and mellicitine) was found to be the principal norditerpenoid toxin extract from *D. brownii*, producing mortality in a broad spectrum of insects (Jennings *et al.,* 1986; Nambi Aiyar *et al.,* 1979). The endogenous function of MLA is unknown, but it has been proposed that it may have a role in protecting the plants against insect damage (Sattelle *et al.,* 1989).

MLA has been shown to be a molecule capable of discriminating between subclasses of nAChR (Table 3.1). It is a very potent inhibitor of αBgt binding nAChRs of the brain (Wonnacott *et al.,* 1993; Ward *et al.,* 1990). In rat brain, MLA inhibits αBgt binding with an IC_{50} of 3.3nM,

compared to an IC_{50} of 8.3μM for inhibition of nicotine binding (Macallan *et al.,* 1988). This indicates that MLA binds preferentially to the αBgt sensitive α7/α8 containing nAChRs, as opposed to the nicotine sensitive α4β2 containing nAChRs. Work on *Xenopus* oocyte expressed homomeric α7 nAChR indicates that MLA is at least as potent at this nAChR, as it is in the rat brain αBgt binding nAChR (Wonnacott *et al.,* 1993)

MLA does not affect hippocampal muscarine, GABA or glutamate receptors (Alkondon *et al.,* 1992; Ward *et al.,* 1990). This emphasises MLA's receptor specificity. MLA is believed to be a competitive antagonist for two principal reasons:

1) In saturation binding assays to hippocampal membranes using [^{125}I]-αBgt, MLA increased the nAChR's k_d for the radioligand (ie. decreased the receptor's affinity for [^{125}I]-αBgt) without increasing the B_{max}. This is characteristic of a competitive antagonist (Wonnacott *et al.,* 1993).

2) In voltage-clamp electrophysiology, MLA produced a voltage independent blockade of the nAChR. This is similar to the blockade produced by αBgt, a competitive antagonist (Sattelle *et al.,* 1989; David & Sattelle, 1984).

MLA can be made semi synthetically from lycoctonine by the addition of a 2-(methylsuccinimido) benzoate ester (Figure 3.3) (Blagbrough *et al.,* 1994b). MLA, lycoctonine and aconitine share a similar norditerpenoid core and consequently all 3 have the ability to block αBgt binding in the brain (Table 3.2). However, the potency of the molecule lies in 2-(methylsuccinimido) benzoate ester (Hardick *et al.,* (in press)).

3.3. Results

3.3.1. Radioligand Binding Assay

αBgt was iodinated as described in section 2.2.13.1. Iodination of αBgt suitable for use in these studies would give >90% incorporation of the ^{125}I label into the αBgt and a specific activity, for the [^{125}I]-αBgt, of ≅700Ci/mmol. An example of an iodination experiment is shown in Figure 3.4. Preliminary experiments were carried out using the COS-7 expression system.

The transfection protocol was optimised by varying the incubation time of the DNA/transfection reagent complex with the cells, then assaying for the % loss in the number of viable cells. Incubation of untransfected and sham-transfected cells (ie. sterile H_2O replaced the DNA in the transfection protocol) in serum free medium, for 6 hours, led to a 3-4% increase in cell death in both cases over cells incubated in $DMEM_g$ and $DMEM/F12_g$. The level of cell death remained the same for transfected cells incubated in serum free medium, for 6 hours (ie. 3-4%). However, if transfected and sham transfected cells were incubated in serum free media for longer than 6 hours, the cell mortality rose to between 12-17%. Whereas, the cell mortality of untransfected cells incubated under the same conditions, only rose to 6%. This indicates that the transfection reagent could be cytotoxic, but that the cytotoxicity was linked to the duration of cell exposure to the reagent. Consequently, 6 hours was taken to be the standard incubation time for the transfection COS-7 and HEK-293 cells.

The [^{125}I]-αBgt binding assay protocols of Gu *et al.,* (1990) and Chavez *et al.,* (1992) both used a volume of 200μl for their radioligand incubation solutions. During this study, preliminary experiments showed that an assay volume of 200μl was not sufficient to cover the bottom of one 35mm diameter well of a 6-well plate, even with gentle agitation. Using volumes of 300μl, 400μl and 500μl of $DMEM_a$ or $DMEM/F12_a$ it was demonstrated that a volume of 500μl was sufficient to cover the bottom of a 35mm diameter well, with gentle agitation.

Once it was established that the assay could detect specific binding of toxin, the length of incubation with the radioligand was optimised. Alkondon & Albuquerque, (1991) showed that maximal blockade of ACh responses in hippocampal neurones, expressing the α7 nAChR, by αBgt took 50-70 minutes. Transfected cells were incubated for between 60-90 minutes (ie. incubation lasted 90 minutes with samples taken every 10 minutes after 60 minutes) with [^{125}I]-αBgt. There was no statistical significant difference between the levels of specific toxin binding seen across this time period (Figure 3.5a). Thus the assay incubation volume was 500μl and the assay incubation time was 60 minutes. These assay parameters were used throughout this study.

Untransfected and sham transfected cells showed no specific binding of [^{125}I]-αBgt. The binding assay, when performed on these cells, consistently exhibited greater non specific binding counts per minute (cpm) than total binding cpm (by between ≅ 300cpm and 2000cpm, depending on the age of the iodinated toxin). Such results were taken to demonstrate the

absence of specific [^{125}I]-αBgt binding throughout this study. Cells transfected with α7 cDNA occasionally showed low levels of specific toxin binding, with an average of 4.6 fmol/mg protein (Figure 3.5b). Hexamethonium was then added to the post-transfection medium, to determine whether this channel blocking drug could improve the expression of the α7 nAChR and hence increase the amount of specific toxin binding seen. A range of concentrations from 10μM to 500μM, was employed. The inclusion of hexamethonium, up to a concentration of 50μM, more than doubled the specific binding of toxin to cells transfected with the α7 nAChR cDNA (Figure 3.6a). With the inclusion of 50μM hexamethonium, the specific binding was 10.72 fmol/mg protein. At concentrations greater than 50μM, the levels of detectable specific binding were not significantly different from that seen with 50μM Hexamethonium. (Figure 3.6a).

The nAChR open channel blocker, dTC, was also employed to assess its ability to modulate α7 nAChR expression (Figure 3.6b). A concentration of 10μM was used, this is well above the IC_{50} of the drug on homomeric and native α7 nAChRs (see Table 1.4). When 10μM dTC was included in the post-transfection medium, the specific toxin binding increased to 14.8 fmol/mg protein. As a result of these data from the COS-7 cells, 10μM dTC was used in all subsequent COS-7 and HEK-293 cell expression experiments.

A chimaeric receptor made from the extracellular N-terminal region of the α7 nAChR and the complementary C-terminal region of the $5HT_3$ serotonin receptor, was used as a control in both expression systems.

Unlike the α7 nAChR, the $5HT_3$ receptor is not permeable to Ca^{2+} ions, but is in fact blocked by them (Eisele *et al.,* 1993; Maricq *et al.,* 1991). Hence, the chimaeric α7-$5HT_3$ receptor should display specific binding similar to that of the α7 nAChR expressed in the presence of antagonists (Figure 3.7). However, the addition of 10μM dTC to HEK-293 cells expressing the chimaeric α7-$5HT_3$ did not significantly increase the level of specific toxin binding.

The HEK-293 expression system displayed greater specific toxin binding of [^{125}I]-αBgt than the COS-7 expression system, when transfected under the same conditions. The difference in expression was ≅40-fold when expression in the presence of dTC was compared. Specific toxin binding to cells expressing the α7-$5HT_3$ receptor was greater, in both systems, than that exhibited by cells transfected with α7 nAChR cDNA alone.

3.3.2. MLA Inhibition

MLA is an α7 nAChR specific competitive antagonist. Thus, if MLA inhibits the specific binding of [^{125}I]-αBgt to cells purporting to express α7 nAChRs, it could be concluded that the receptors are indeed the product of the transfecting α7 cDNA. The experiments were carried out in both expression systems, with 10μM dTC included in the post-transfection medium (Figure 3.8). The radioligand binding assay was modified by the inclusion of increasing concentrations of MLA (10pM to 1μM) in the 'total' and 'non-specific' binding solutions. The MLA inhibited the specific binding of the toxin in a saturable dose dependent manner, with a mean IC_{50} of 9.9±6.9nM in the

COS-7 expression system and 0.9±0.1nM in the HEK-293 expression system. Both values compare well to MLA's reported IC_{50} at α7 nAChRs (Table 3.1). It is not known whether the differences in the IC_{50}'s are intrinsic to the expression systems. However, these data suggest that the transfected cells are expressing receptors with a distinct α7 nAChR character.

3.3.3. FITC-αBgt Binding Assay

The radioligand binding assay detected surface αBgt binding. The employment of αBgt conjugated to the fluorescence label FITC, should permit the visualisation of this binding. Figures 3.9a-3.9d shows photomicrographs of untransfected and transfected COS-7 cells labelled by FITC-αBgt, under total and non-specific binding conditions. Untransfected cells displayed little difference between total and non-specific toxin binding (Figure 3.9a). Similar levels of toxin binding was seen in cells transfected with α7 nAChR cDNA in the absence of 10μM dTC (Figure 3.9b).

The inclusion of 10μM dTC in the post-transfection medium of α7 nAChR cDNA transfected cells, produced intense total binding fluorescence (≅98% cells labelled; Figure 3.9c). The presence of excess unlabelled αBgt produced levels of non-specific binding, in these cells, reminiscent of that displayed in Figures 3.9a & 3.9b. These results show that greater levels of toxin specifically bound to cells grown in the presence of dTC, than to cells grown in the drug's absence. The FITC-αBgt binding to COS-7 cells transfected with the chimaeric α7-$5HT_3$ receptor cDNA (Figure 3.9d), was at a level comparable to that seen in Figure 3.9c. These labelling

data confirm the findings recorded for the radioligand binding assay. Specific toxin binding is increased in cells transfected with α7 nAChR cDNA in the presence of channel blocking antagonists, in a manner similar to that seen in cells transfected with the Ca^{2+} impermeable chimaeric α7-$5HT_3$ receptor cDNA.

3.4. Discussion

The lack of specific binding of αBgt, detected by the radioligand and fluorescence binding assays, to untransfected COS-7 and HEK-293 cells, indicates the absence of endogenous surface expressed αBgt binding nAChRs. Thus specific binding of [^{125}I]-αBgt or FITC-αBgt detected on transfected cells, from either of these 'naive' cell lines, would be due to the product of the transfecting cDNA. COS-7 cells transfected with α7 nAChR cDNA occassionally exhibited increased, but low levels of specific toxin binding when compared to untransfected cells and other αBgt binding cell lines (Chini *et al.,* 1992) (Figure 3.7). This suggested that the product of the α7 nAChR cDNA (ie. homomeric α7 nAChRs) was reaching the surface of these transfected cells and also, that the specific toxin binding of 4.6 fmol/mg protein (Figure 3.5b) was too low to be detected visually by the fluorescence binding assay (Figure 3.9b). This final suggestion is unsurprising, considering reports of iodine radioligand assays being as much as 1500 times more sensitive than comparable fluorescence assays (Nairn, 1976).

A previous transient expression study, reported no detectable surface expression of the α7 nAChR (Chen & Patrick, 1993). They used anti-

α7 antibodies in western blots, to demonstrate that the α7 nAChR protein was present in the transfected cells. This indicates a possible problem in the nascent α7 nAChR's membrane translocation mechanism. However, the protein detected was present at such low levels that if it did reach the cell's surface, it was probably below the sensitivity of conventional ligand binding techniques. This was probably the reason for the failure to detect specific toxin binding in some of the early expression experiments (Figure 3.7). But why should the expression of α7 nAChRs in cultured cells be so low, when compared to the expression of other neuronal nAChRs in cultured cells (eg. M10 cell line for the α4β2 nAChR; Whiting *et al.,* 1991)

Ca^{2+} cytotoxicity, caused by α7 nAChRs high Ca^{2+} permeability (Seguela *et al.,* 1993; Vijayaraghavan *et al.,* 1992), could be a reason for the low levels of specific toxin binding. In the case of cultured cells transfected glutamate receptor cDNA, low expression and binding was due to Ca^{2+} excitotoxicity (Tymianski *et al.,* 1993a; Roth & Olney, 1987). Constituents within the cells' culture medium activated the ion channel and permitted the entry of unregulated Ca^{2+}. This led to poor receptor expression and ultimately cell death, through mechanisms possibly involving VDCCs and Ca^{2+} dependent secondary messenger pathways (Tymianski *et al.,* 1993c). This was overcome by three methods; the addition of antagonists, point mutations at the Q/R/N site within the putative reentrant M2 region of the receptor and the use of glutamate/glutamine free medium (Bennett & Dingledine, 1995; Cik *et al.,* 1994; Tygesen *et al.,* 1994; Mori *et al.,* 1992).

DMEM and DMEM/F12 are 'defined' media and do not contain constituents recognised to activate α7 channels. However, the growing medium used was 'complex', due to its supplementation with 10% (v/v) FCS (see $DMEM_g$ and $DMEM/F12_g$ in Chapter 2). Hence, there exists the possibility of constituent(s) of FCS, as yet unidentified, being capable of activating α7 nAChRs and permitting Ca^{2+} entry. The constituent(s) need not bind to the ACh site like conventional nicotinic agonists. They may bind elsewhere on the N-terminal extracellular domain like the acetylcholinesterase antagonist and nicotinic agonist, physostigmine/eserine (Lena & Changeux, 1993; Schrattenholz *et al.,* 1993). Physostigmine is proposed to bind to neuronal nAChRs in the region of K125 and activate the ion channel, even in the presence of desensitising concentrations of ACh (Pereira *et al.,* 1993; Kuhlmann *et al.,* 1991). The binding of physostigmine is not inhibited by αBgt, MLA and dTC (Kuhlmann *et al.,* 1991; Okonjo *et al.,* 1991). The existence of a 'physostigmine like' channel activator could provide and explanation for the previous low toxin binding data, seen in cells expressing homomeric α7 nAChRs and grown in the presence of desensitising concentrations of nicotine (ie. a concentration of nicotine that normally induces an agonist closed form of the receptor; J-L. Galzi, personal communication). Thus it would be possible for unregulated Ca^{2+} to enter cells transfected with α7 nAChR cDNA, in the absence of what would be considered a conventional nicotinic agonist. This ligand gating of the ion channel could be further exacerbated by Ca^{2+}, which is known to allosterically potentiate and increase the probability of opening α7 nAChRs (Eisele *et al.,*

1993; Vernino *et al.,* 1992). It is possible that this channel activator, like physostigmine, stabilises an as yet unidentified active conformational state of the nAChR. In this state the ion channel is open with the ACh binding site empty. However, the binding site still retains its ability to bind conventional nicotinic agonists and antagonists. If correct, a further modification to the allosteric transition model proposed in Figure 1.4 would have to be made. The effectiveness of the non-competitive anaesthetic dibucaine at inhibiting physostigmine activated channels, indicates that channel blockers may be employed to improve the specific toxin binding (Okonjo *et al.,* 1991).

The channel blocking antagonist hexamethonium was included in the post-transfection medium of COS-7 cells. This drug could restrict Ca^{2+} entry through the channel. Hexamethonium increased the observed specific toxin binding in a concentration dependent manner, with the greatest increase seen at 50μM (Figure 3.6a). At higher concentrations of hexamethonium, the levels of specific toxin binding seen were not statistically different to that exhibited at 50μM hexamethonium. This may have been due to prolonged exposure of the cells to the drug at these concentrations possibly being cytotoxic. The polymethylene bistrimethylammonium salt family, to which hexamethonium belongs, are also known to have anticholinesterase and antibacterial activity. Hence, hexamethonium may interact with cellular functions resulting in the decrease of specific toxin binding. It should be noted that 50μM is ≅40 times less than the IC_{50} reported for hexamethonium at homomeric α7 nAChRs (Anand *et al.,* 1993). However, the injected *Xenopus* oocytes expressing these receptors are not incubated

with these greater concentrations hexamethonium for more than a few minutes.

The inclusion of 10μM dTC in the post-transfection medium of transfected COS-7 cells, further increased the specific toxin binding to 14.8 fmol/mg protein. This level of [^{125}I]-αBgt binding was similar to that seen in SK-N-BE neuroblastoma cells, that express native α7 nAChRs (Chini *et al.,* 1992). It has been demonstrated that dTC inhibits nAChRs via 2 concentration dependent routes: as a competitive antagonist at concentrations of ≅1μM; as a non-competitive channel blocking antagonist at concentrations >5μM (Shaker *et al.,* 1982). At a concentration of 10μM, dTC should be acting, like hexamethonium, as a channel blocker. If dTC and hexamethonium are working in a similar fashion, the relative increases in specific toxin binding could be explained by the drugs' IC_{50}s. With an IC_{50} of 0.27μM at the homomeric α7 nAChR, 10μM dTC would have a greater blockading effect than 50μM hexamethonium with an IC_{50} of 2mM at the same receptor. However, the data could be simply explained by dTC being a more efficacious drug than hexamethonium.

HEK-293 cells transfected with α7 nAChR cDNA displayed specific toxin binding of 112.9 fmol/mg protein (Figure 3.7). This level of binding lies in between the 90 fmol/mg protein, seen in SH-SY5Y neuroblastoma cells, and the 150 fmol/mg protein, seen in cultured hippocampal neurones and IMR32 neuroblastoma cells, all expressing native αBgt binding nAChRs (Barrantes *et al.,* 1995; Chini *et al.,* 1992). The inclusion of 10μM dTC in the HEK-293 cells' post-transfection medium,

increased the specific toxin binding by ≅5-fold. This result suggests that the increased specific toxin binding seen in both expression systems, in the presence of dTC, could be due to the inhibition of the entry of cytotoxic levels of Ca^{2+}. Further evidence of specific toxin binding being linked to Ca^{2+} permeability, was possibly supported when COS-7 and HEK-293 cells were transfected with the chimaeric α7-$5HT_3$ receptor cDNA.

Ca^{2+} impermeability is a feature of the $5HT_3$ receptor's ion channel that the chimaera possesses (Eisele *et al.,* 1993). However, it should be noted that a recent report proposed that homomeric $5HT_3$ receptors are Ca^{2+} permeable (Hargreaves *et al.,* 1994). This conflicts with the previous data on homomeric $5HT_3$ receptors, reported by Eisele *et al.,* (1993), Maricq *et al.,* (1991) and Henderson, (1990), that they are Ca^{2+} impermeable. Specific toxin binding was ≅2.5 times greater in COS-7 cells and ≅3.5 times greater in HEK-293 cells, than the binding seen in the cells transfected with α7 nAChR cDNA alone (Figure 3.7). Based on the reports of Eisele *et al.,* (1993) and Maricq *et al.,* (1991), this level of specific toxin binding could be attributable to the chimaera's Ca^{2+} impermeability. However, another explanation for the level of specific toxin binding seen in cell transfected with the chimaeric α7-$5HT_3$ receptor, could be that it is expressed and translocated to the membrane more efficiently than the α7 nAChR. Thus the level of expression of the chimaeric α7-$5HT_3$ seen in this study, would be independent of Ca^{2+} permeability (see Hargreaves *et al.,* 1994). If this is so the premiss that unregulated cytotoxic Ca^{2+} was the cause for the low levels of specific toxin binding, was incorrect. Evidence for this comes from the fact

that Ca^{2+} cytotoxicity is associated with a decrease in the total protein concentration and the number of viable cells within a culture (Cik *et al.,* 1994). In this study total protein concentrations were between 0.4-0.45mg/35mm dish for COS-7 cells and 0.3-0.45mg/35mm dish for HEK-293 cells (transfected cells, transfected cells in the presence of antagonists and untransfected cells). Thus cell death does not seem to be taking place, otherwise total protein levels in cells transfected with α7 cDNA alone would have been much lower. Hence, an alternative explanation for the increases in specific toxin binding, seen when antagonists were included in the post-transfection medium, could be proposed.

The presence of antagonists may, through a mechanism such as that proposed below, lead to more efficient surface expression/upregulation of the α7 nAChR. *In vivo* and *in vitro* exposure of tissues expressing neuronal nAChRs to desensitising levels of agonist, leads to an upregulation in the numbers of expressed receptors (Barrantes *et al.,* 1995; Flores *et al.,* 1992; Wonnacott, 1990). Upregulation seems to require the allosteric transition of the receptor from the 'resting' to 'desensitised' states, to go via the 'activated' state (Barrantes *et al.,* 1995; Schwartz & Kellar, 1985). In this desensitised state, the receptors are functionally blocked and upregulation is proposed to occur through decreased receptor turnover (ie. via decreased rates of surface receptor co-option and degradation) (Peng *et al.,* 1994). The channel activating ligand in the growth medium may, like physostigmine, only activate the nAChR without inducing desensitisation (Pereira *et al.,* 1993). Thus, the ligand on its own could not

produce an upregulation in receptor number. The binding of an antagonist that stabilises the 'desensitised' state of the receptor, could have the same 'upregulation' effect as prolonged exposure to agonist. dTC stabilises the 'desensitised' state of the nAChR (Lena & Changeux, 1993). The α7 nAChR expressed in cultured cells and in the presence of 10μM dTC, could have undergone the allosteric transitions necessary to produce 'agonist induced like' receptor upregulation. Hence, as well as by blocking the channel, dTC could also be increasing the level of specific toxin binding by inducing receptor upregulation. The effect of binding hexamethonium on the nAChR conformation is unclear. The lower effectiveness of hexamethonium compared to dTC may be explained by this proposed mechanism if, like MK801, hexamethonium's blocking of the channel induces little or no conformational change in the nAChR (Stephens, 1994; Ramoa *et al.,* 1990). The data from HEK-293 cells expressing the chimaeric α7-5HT$_3$ in the presence of dTC, suggests that this mechanism may be unique to the α7 nAChR.

The validity of this theory could be tested by ligand binding and whole cell voltage-clamp recording experiments. The expected results would show an increase in B_{max}, but no change in the K_d and a decrease in the level of receptor function. The presence of the 'allosteric channel activator' could be screen for using patch clamped α7 nAChR expressing hippocampal neurone membranes (like Pereira *et al.,* (1993) when assaying the channel activating ability of physostigmine) or membranes from *Xenopus* oocytes expressing the homomeric α7 nAChR.

However, this transient expression system has determined the parameters within which a stably transfected cell line, expressing the homomeric α7 nAChR, can be developed.

The HEK-293 expression system exhibited significantly greater specific toxin binding than that seen in the COS-7 expression system, when transfected under the same conditions. The reasons for this are unknown. Statistical comparisons between the expression systems' % changes in specific toxin binding (Figure 3.7), are not significant (with a confidence threshold probability of 0.05). Therefore the relative levels of specific toxin binding, seen in both expression systems, are statistically similar. This suggests that dTC increases the expression of α7 nAChR in COS-7 cells and HEK-293 by the same extent. This is mirrored by the statistically similar relative levels of specific toxin binding, exhibited by cells transfected with chimaeric α7-5HT_3 cDNA. These results indicate intrinsic differences between the expression systems, rather than in the receptor expressed. Further evidence of this was provided when the preliminary experiments using the HEK-293 cells were performed, using α7 nAChR cDNA in the pRc/CMV vector. The results of the toxin binding assays (not shown) were statistically similar to those subsequently produced by the cells using α7 nAChR cDNA in the pMT3 vector. Hence, this would indicate that the HEK-293 cells are better expressers of the α7 nAChR than COS-7 cells. This may be due to the HEK-293 cells' maintenance of a higher vector copy number than the COS-7 cell or just a more efficient exogenous protein expression apparatus (ie. better transcription, translation, membrane translocation and

mechanisms). Northern blots or permeabilisation of the cells to look at the numbers of receptors in transit to and from the membrane surface, may help to reveal more about the differences in expression between the cell lines.

The nicotinic nature of the αBgt binding detected was confirmed by the binding of MLA. MLA inhibited specific toxin binding in both expression systems with IC_{50}'s in the same range as those previously reported for the α7 nAChR. This suggests that the transfected cells COS-7 and HEK-293 cells are expressing the purported homomeric α7 nAChR. However, if both expression systems are expressing statistically similar receptors, the IC_{50}'s should also be similar. The sources and length of storage of the MLA samples used during this study could provide a possible explanation. The MLA sample used in the COS-7 cell experiments, was slightly older and from a different batch from the sample used in the HEK-293 experiments. This older MLA sample may have chemically degraded to a less potent form. When used experimentally, this sample would give an apparent impression that the α7 nAChRs expressed in COS-7 cells, were less sensitive to MLA than those expressed in the HEK-293 cells. Hence, providing a possible explanation for the differences in the IC_{50}'s (Figure 3.8). The MLA inhibition of specific toxin binding to homomeric α7 nAChRs expressed in COS-7 cells, should be repeated with the same batch of MLA used in the HEK-293 expression experiments and with extra concentration points in the middle of the curve. However, the data from this study may provide the first reported IC_{50} for MLA for the homomeric α7 nAChR expressed in a mammalian cell line.

The fluorescence labelling of transfected COS-7 cells (Figure 3.9) gave visual confirmation of the radioligand binding results. The inclusion of 10μM dTC in the post-transfection medium or transfection with the Ca^{2+} impermeable chimaeric receptor cDNA, led to an increase in the number and intensity of FITC-αBgt labelled cells. Thus displaying visually the increase in the number of surface expressed homomeric α7 nAChRs. The proportion of labelled cells is consistent with the report by Felner *et al.,* (1987), demonstrating the potential of the transfection agent/DNA complex, when employed in proportions similar to that above, to interact with virtually all the cells to be transfected. Preliminary binding experiments performed on HEK-293 cells has exhibited similar results (results not shown).

3.4.1. Summary

COS-7 and HEK-293 cells, when transfected with chick α7 nAChR cDNA, express low levels of putative homomeric α7 nAChRs. The expression of the receptor, in both COS-7 and HEK-293 cells, was increased by the addition of antagonists. Hexamethonium and dTC, acting as non-competitive channel blockers, are believed to inhibit the entry of cytotoxic levels of Ca^{2+} in the cells. The belief that unregulated Ca^{2+} influx was responsible for the low α7 nAChR expression, was possibly supported by the higher levels of specific [^{125}I]-αBgt binding seen in transfected cells expressing the Ca^{2+} impermeable chimaeric α7-$5HT_3$ receptor. However, more efficient expression and 'conformation-induced' upregulation are alternative explanations for the increased levels of specific toxin binding seen

in the cultured cells. The α7 homomeric nAChRs were further characterised by MLA. This potent α7 specific alkaloid inhibited specific toxin binding in a saturable concentration dependent manner. The IC_{50} for MLA was similar to that reported for it at other α7 nAChRs. The use of FITC-αBgt permitted the fluorescent visualisation of the radioligand binding results. The inclusion of dTC in the post-transfection medium, resulted in an increase in the number and intensity of fluorescence labelled cells. The data also showed the HEK-293 expression system to be statistically more efficient than the COS-7 expression system.

Figure 3.1

The cDNA and amino acid sequence of chick α7. The *Hin*DIII (AAGTTC), *Pst*I (CTGCAG), *Bam*HI (GGATCC), *Xba*I (TCTAGA) and *Not*I (GCGGCCGC) sites used for subcloning are marked in *italics*. The signal peptide, Cys loop and vicinal cysteines are marked in **bold**. The four putative transmembrane domains are underlined. The use of the *Eco*RI (GAATTC, nucleotides 739 to 744) and *Bgl*II (AGATCT, nucleotides 902 to 913) sites will be discussed later (marked in ***bold italics***).

HinDIII ***PstI***

*AAGCTT*GGG*CTGCAG*GTCGAAATTCGGCACGAGAGCCGACATCGCAGGGAGAGCCCCGCG
10 20 30 40 50 60

CGGGCGCAGGAGGGGGCGGGGCGGCGAGAGGTCCGCGGCGGCGACGGAGGCTCCCGGCTC
70 80 90 100 110 120

M G L R A L M L W L L A A 13
CGGCTGCTGCGGCTCCCGGAATGGGCCTCCGGGCGCTGATGCTGTGGTTGCTGGCGGCGG
130 140 150 160 170 180

A G L V R E S L Q G E F Q R K L Y K E L 33
CGGGGCTCGTGCGCGAGTCCCTGCAAGGAGAGTTCCAAAGGAAGCTGTACAAGGAGCTGC
190 200 210 220 230 240

L K N Y N P L E R P V A N D S Q P L T V 53
TGAAGAACTACAACCCTCTGGAACGACCAGTTGCAAATGACTCCCAGCCGCTCACTGTCT
250 260 270 280 290 300

Y F T L S L M Q I M D V D E K N Q V L T 73
ATTTCACTCTCAGCCTCATGCAGATCATGGATGTGGATGAAAAGAATCAAGTATTAACAA
310 320 330 340 350 360

T N I W L Q M Y W T D H Y L Q W N V S E 93
CAAACATCTGGCTACAAATGTACTGGACAGATCATTACTTACAGTGGAATGTGTCTGAAT
370 380 390 400 410 420

Y P G V K N V R F P D G L I W K P D I L 113
ACCCTGGAGTGAAGAACGTCCGTTTTCCTGATGGACTGATTTGGAAGCCAGATATTCTTC
430 440 450 460 470 480

L Y N S A D E R F D A T F H T N V L V N 133
TCTATAACAGTGCTGATGAAAGATTTGATGCTACATTTCACACTAATGTTTTAGTCAATT
490 500 510 520 530 540

S S G H C Q Y L P P G I F K S S **C Y I D** 153
CTTCGGGACACTGCCAATATCTGCCACCAGGCATATTTAAAAGCTCATGCTACATAGACG
550 560 570 580 590 600

V R W F P F D V Q K C N L K F G S W T Y 173
TGCGTTGGTTTCCATTTGATGTTCAGAAGTGCAATCTGAAGTTTGGATCTTGGACATATG
610 620 630 640 650 660

G G W S L D L Q M Q E A D I S G Y I S N 193
GAGGCTGGTCCTTAGACTTACAAATGCAAGAAGCAGATATATCCGGCTATATTTCAAATG
670 680 690 700 710 720

G E W D L V G I P G K R T E S F Y E **C C** 213
GAGAGTGGGATTTAGTAG***GAATTC***CTGGGAAGAGAACTGAGAGCTTTTATGAGTGCTGTA
730 740 750 760 770 780

K E P Y P D I T F T V T M R R R T L Y Y 233
AAGAACCATACCCAGATATCACATTCACAGTAACCATGAGACGCAGAACTCTCTACTACG
790 800 810 820 830 840

G L N L L I P C V L I S A L A L L V F L 253
GGCTCAACCTTCTTATTCCCTGTGTACTGATATCAGCACTTGCCTTATTAGTCTTTCTGC
850 860 870 880 890 900

```
L P A D S G E K I S L G I T V L L S L T          273
TTCCAGC***AGACTC***AGGAGAAAAGATCTCACTAGGTATAACAGTTTTATTGTCTCTCACCG
       910        920        930        940        950        960

V F M L L V A E I M P A T S D S V P L I          293
TCTTCATGTTACTCGTGGCTGAAATTATGCCAGCAACATCTGATTCTGTGCCCTTAATTG
       970        980        990       1000       1010       1020

A Q Y F A S T M I I V G L S V V V T V I           313
CVCAGTATTTTGCCAGCACCATGATTATTGTTGGCCTCTCTGTTGTTGTCACTGTTATCG
      1030       1040       1050       1060       1070       1080

V L Q Y H H H D P D G G K M P K W T R V           333
TTCTACAATACCATCATCACGATCCAGATGGGGGAAAAATGCCTAAATGGACAAGAGTCA
      1090       1100       1110       1120       1130       1140

I L L N W C A W F L R M K R P G E D K V           353
TCCTTCTGAATTGGTGTGCTTGGTTTCTGAGGATGAAGAGACCAGGGGAAGATAAAGTGC
      1150       1160       1170       1180       1190       1200

R P A C Q H K Q R R C S L S S M E M N T           373
GTCCCGCCTGTCAACATAAACAGCGTCGATGCAGCCTGTCAAGCATGGAGATGAACACTG
      1210       1220       1230       1240       1250       1260

V S G Q Q C S N G N M L Y I G F R G L D           393
TGAGTGGTCAGCAATGCAGTAATGGGAACATGCTGTATATTGGGTTTCGAGGGCTGGATG
      1270       1280       1290       1300       1310       1320

G V H C T P T T D S G V I C G R M T C S           413
GGGTTCACTGCACACCCACCACTGATTCAGGGGTGATCTGTGGGAGGATGACCTGTTCAC
      1330       1340       1350       1360       1370       1380

P T E E E N L L H S G H P S E G D P D L           433
CAACAGAGGAAGAAAATCTTCTGCACAGTGGCCACCCCTCTGAAGGCGACCCAGATTTGG
      1390       1400       1410       1420       1430       1440

A K I L E E V R Y I A N R F R D Q D E E           453
CTAAGATCCTGGAAGAGGTCAGATACATTGCAAACAGGTTCAGAGACCAGGATGAAGAAG
      1450       1460       1470       1480       1490       1500

E A I C N E W K F A A S V V D R L C L M           473
AAGCCATTTGCAACGAATGGAAGTTTGCAGCCTCTGTAGTAGATCGGCTCTGCTTGATGG
      1510       1520       1530       1540       1550       1560

A F S V F T I I C T I G I L M S A P N F           493
CATTTTCGGTCTTCACCATCATTTGTACAATTGGCATCTTAATGTCAGCACCAAACTTTG
      1570       1580       1590       1600       1610       1620

V E A V S K D F A *                               502
TAGAGGCTGTCTCTAAAGATTTTGCTTAACTCCTAACTATGATTTGATTCTCTGAAGTAT
      1630       1640       1650       1660       1670       1680

CATATGTAGCAAATAAGAGTGTATTTTTTTTTTGTTTGTTTGCTTGTTTTTAATGAGTACA
      1690       1700       1710       1720       1730       1740

CTGTATCTCATTGTCCTCTGTCTTTTGCCCCCCCCCCTATTCCCCCAGCATTCCTCTGAC
      1750       1760       1770       1780       1790       1800
```

BamHI ***X***

CCTGGGCTCCTTTCTGAACAGGAGCACCCTTTCAGAAGGGGAGCCAG*GGATCC*ACTAGT*T*

1810 1820 1830 1840 1850 1860

baI ***NotI***

CTAGAGCGGCCGCC

1870

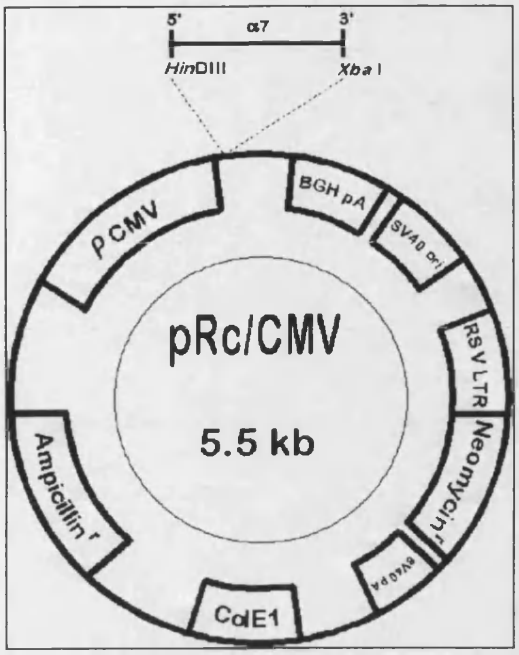


Figure 3.2a

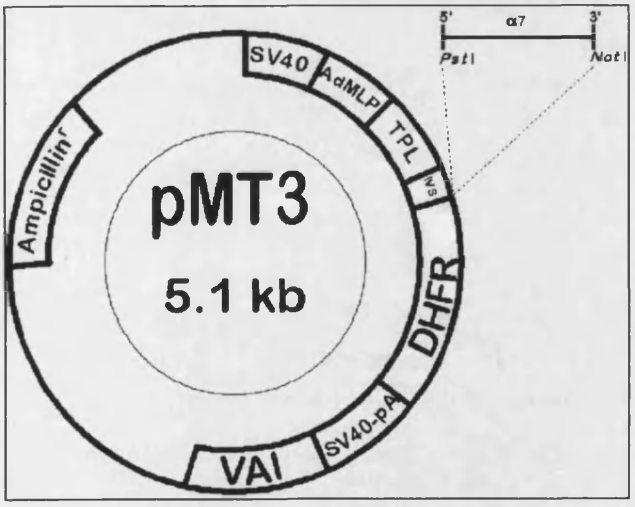
The pRc/CMV vector used in the COS-7 cell expression system.

P CMV	CMV promoter
BGH pA	Bovine growth hormone polyadenylation signal
SV40 *ori*	SV40 origin of replication
RSV LTR	Rous sarcoma virus long terminal repeats
Neomycin^r	Neomycin resistance marker
SV40 pA	SV40 polyadenylation signal
ColE1	Colicinogeny factor E1
Ampicillin^r	Ampicillin resistance marker (β-lactamase)

Figure 3.2b

The pMT3 vector used in the HEK-293 cell expression system.

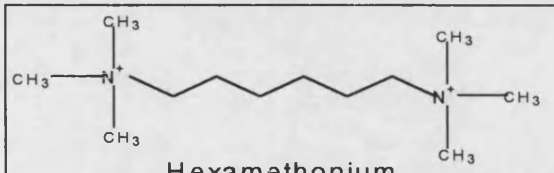
SV40 pA	SV40 polyadenylation signal
VAI	Adenovirus associated inhibitor gene



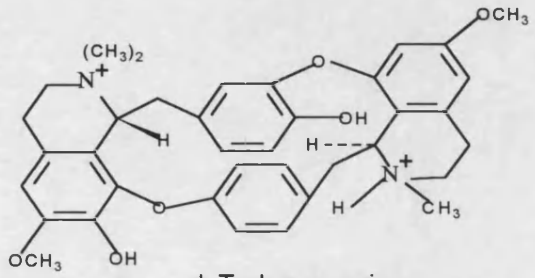
IVS	Intervening sequence
AdMLP	Adenovirus Major late promoter
DHFR	Dihydrofolate Reductase marker
Ampicillin^r	β-lactamase marker
TPL	Adenovirus tripartite leader sequence

Figure 3.3

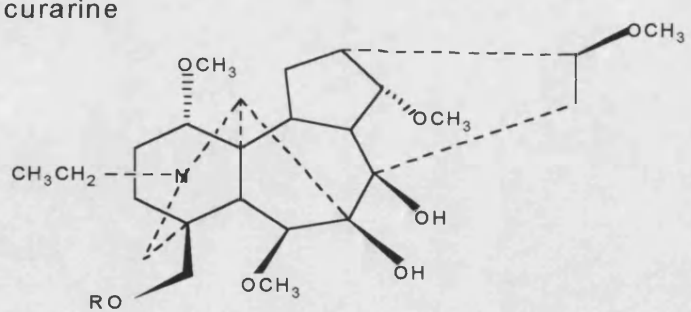
The molecular structures of the nicotinic antagonists hexamethonium, d-tubocurarine, methyllycaconitine (MLA), 3-deoxy-18-*O*-desmethyl(2-aminobenzoyl) aconitine and 3-deoxy-18-*O*-desmethyl[2-(methylsuccinimido) benzoyl] aconitine. Lycoctonine is converted to MLA by the addition of a methylsuccinimido benzoate R group. Aconitine is a voltage gated Na^+ channel activator (Rubin & Soderlund, 1992), modifications to its norditerpenoid core converted it to a potent nicotinic antagonist.



Hexamethonium



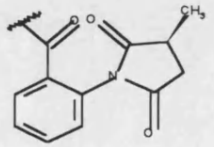
d-Tubocurarine

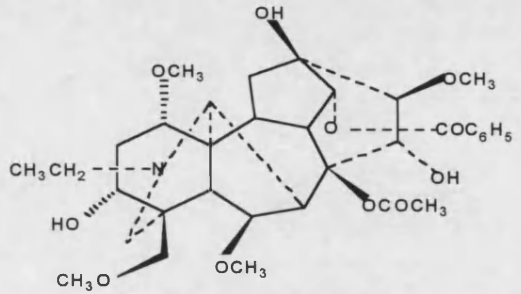


R Group

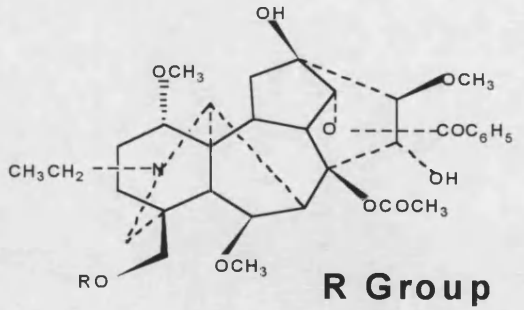
Lycoctonine **H**

MLA

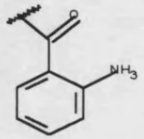




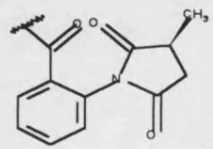
Aconitine



R Group



3-deoxy-18-*O*-desmethyl
(2-aminobenzoyl) Aconitine



3-deoxy-18-*O*-desmethyl
[(methylsuccinimido)-
benzoyl] Aconitine

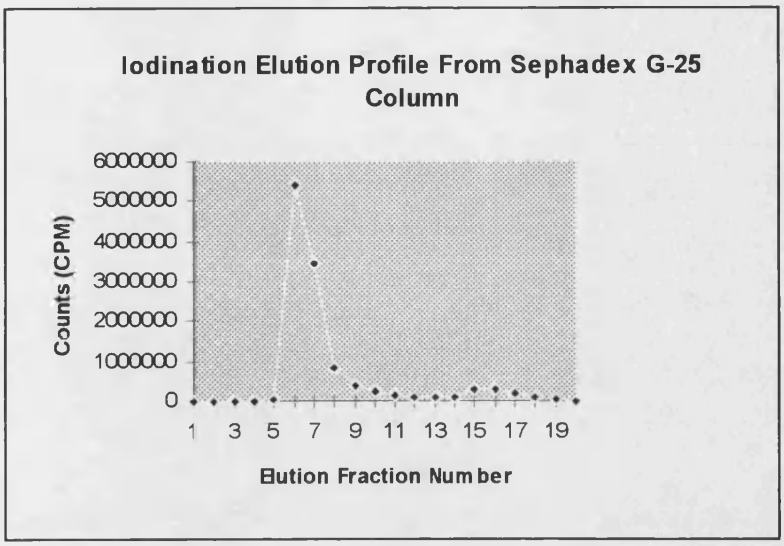


Figure 3.4

Typical results from an experiment to iodinate αBgt (5μl samples of each 1ml fraction were counted).

Σ Total ^{125}I (2mCi; fractions 5-19)	11642683cpm
Σ Total [^{125}I]-αBgt (2.5nmol; fractions 5-13)	10670521cpm
Σ peak [^{125}I]-αBgt (fractions 6-8)	9670143cpm
Σ Free ^{125}I (fractions 14-19)	972162cpm

In [^{125}I]-αBgt peak

Concentration of [^{125}I]-αBgt $= \frac{\sum \text{peak } [^{125}\text{I}]\text{-}\alpha\text{Bgt}}{\sum \text{Total } \alpha\text{Bgt}} \times \frac{2.5\text{nmol}}{3\text{ml}}$

$= \frac{9670143\text{cpm}}{10670521\text{cpm}} \times \frac{2.5\text{nmol}}{3\text{ml}} = 0.76\text{nmol/ml}$

$= \mathbf{0.76\mu M}$

Concentration of ^{125}I in peak $= \frac{\sum \text{peak } [^{125}\text{I}]\text{-}\alpha\text{Bgt}}{\sum \text{Total } ^{125}\text{I}} \times \frac{2\text{mCi}}{3\text{ml}}$

$= \frac{9670143\text{cpm}}{11642683\text{cpm}} \times \frac{2\text{mCi}}{3\text{ml}} = \mathbf{0.55mCi/ml}$

% Incorporation of ^{125}I into αBgt $= \frac{\sum \text{Total } [^{125}\text{I}]\text{-}\alpha\text{Bgt}}{\sum \text{Total } ^{125}\text{I}} \times 100\% = \mathbf{90.62\%}$

Specific activity of [^{125}I]-αBgt $= \frac{\text{Concentration of } ^{125}\text{I in peak}}{\text{Concentration of } [^{125}\text{I}]\text{-}\alpha\text{Bgt in peak}}$

$= \frac{0.55\text{mCi/ml}}{0.76\text{nmol/ml}} = 0.723\text{mCi/nmol}$

$= \mathbf{723Ci/mmol}$

Figure 3.5

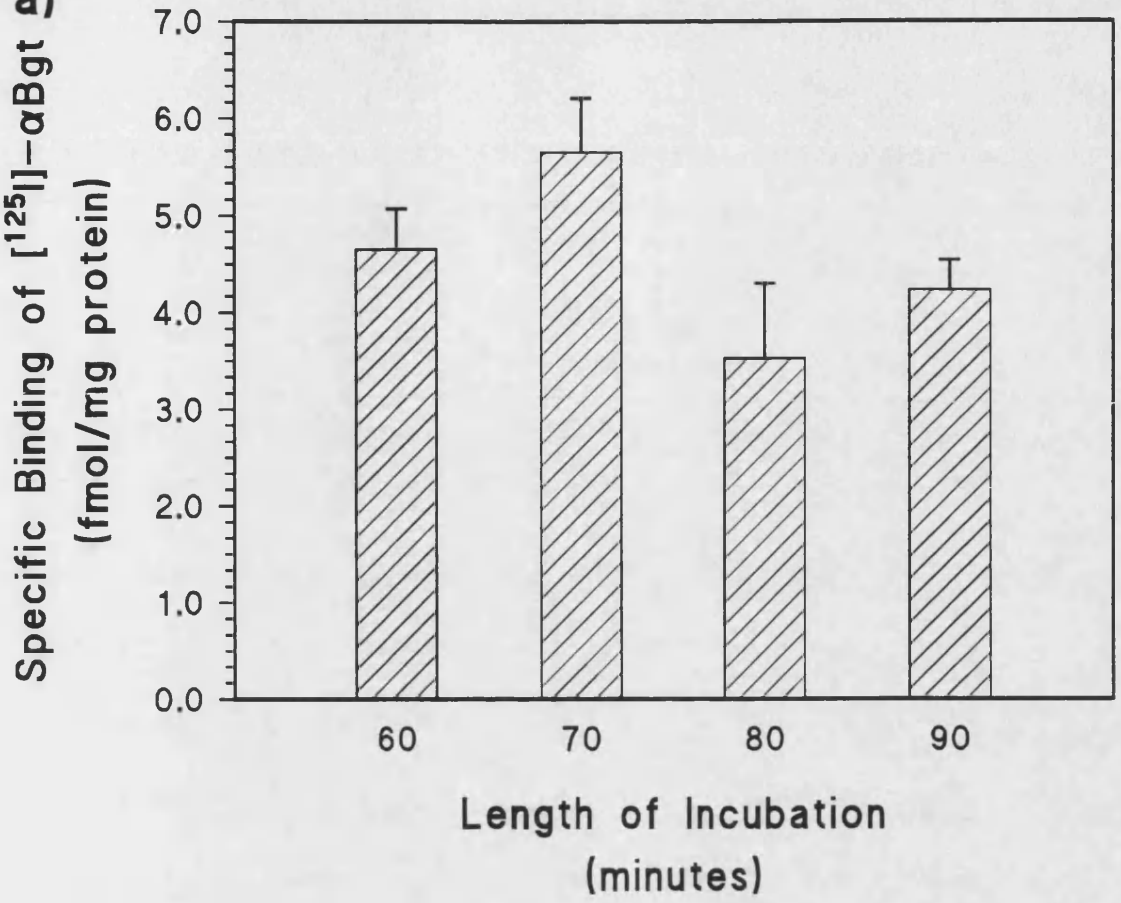
a) The effect of different lengths of incubation on the results of binding assay performed on transfected COS-7 cells. The levels of specific toxin binding detected were shown, by Students' paired *t* test ($P<0.02$), not to be significantly different. Total binding replicate counts were between 7000-14000cpm and non specific replicate counts were between 4000-9000cpm. However, the individual level of replicate counts for both total and non-specific binding displayed more inherent variability, the older the [^{125}I]-αBgt was. Hence, [^{125}I]-αBgt no older than 3 weeks, from date of iodination, was used in this study.

In these experiments n=2 and values are displayed as mean & range. Each value within one experiment consisted of 9 replicates.

b) Results from five [^{125}I]-αBgt specific binding experiments performed on COS-7 cells transfected with α7 nAChR cDNA. None detected indicates that the experiment exhibited non specific binding greater than the total binding (in a fashion similar to that seen in untransfected and sham transfected cells), hence no detectable specific binding of [^{125}I]-αBgt.

Each experiment consisted of 12 replicates.

a)


Specific Binding of [125I]-αBgt
(fmol/mg protein)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
60
70
80
90
Length of Incubation
(minutes)

b)

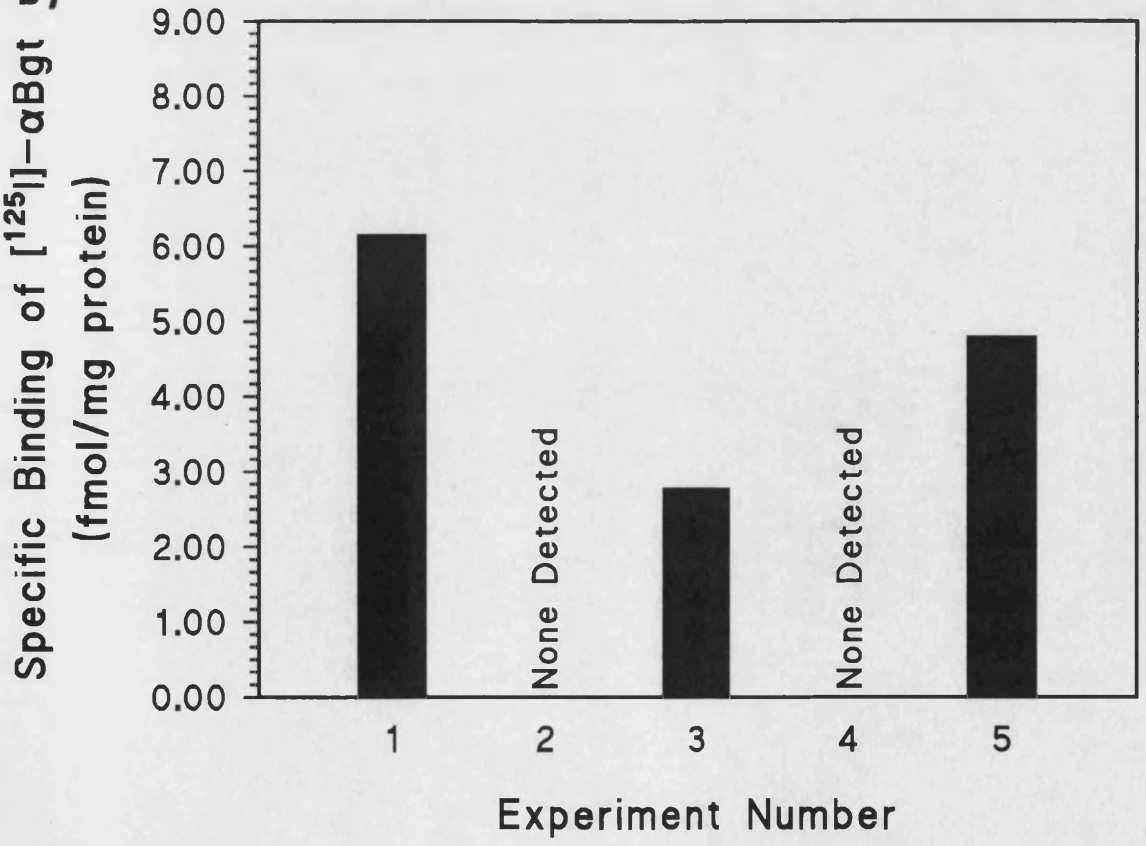

Specific Binding of [125I]-αBgt
(fmol/mg protein)
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
None Detected
None Detected
1
2
3
4
5
Experiment Number

Figure 3.6

a) The effect of including hexamethonium in the post-transfection medium, on the specific binding of [^{125}I]-αBgt to COS-7 cells transfected with α7 cDNA. All concentrations of hexamethonium, with the exception of 10μM, increased specific binding significantly ($P<0.05$) over COS-7 cells transfected with α7 cDNA in the absence of hexamethonium. The specific binding of [^{125}I]-αBgt exhibited by transfected cells in the presence of 10μM hexamethonium was not significantly different, by Students' paired *t* test, from transfected cells in the absence of hexamethonium.

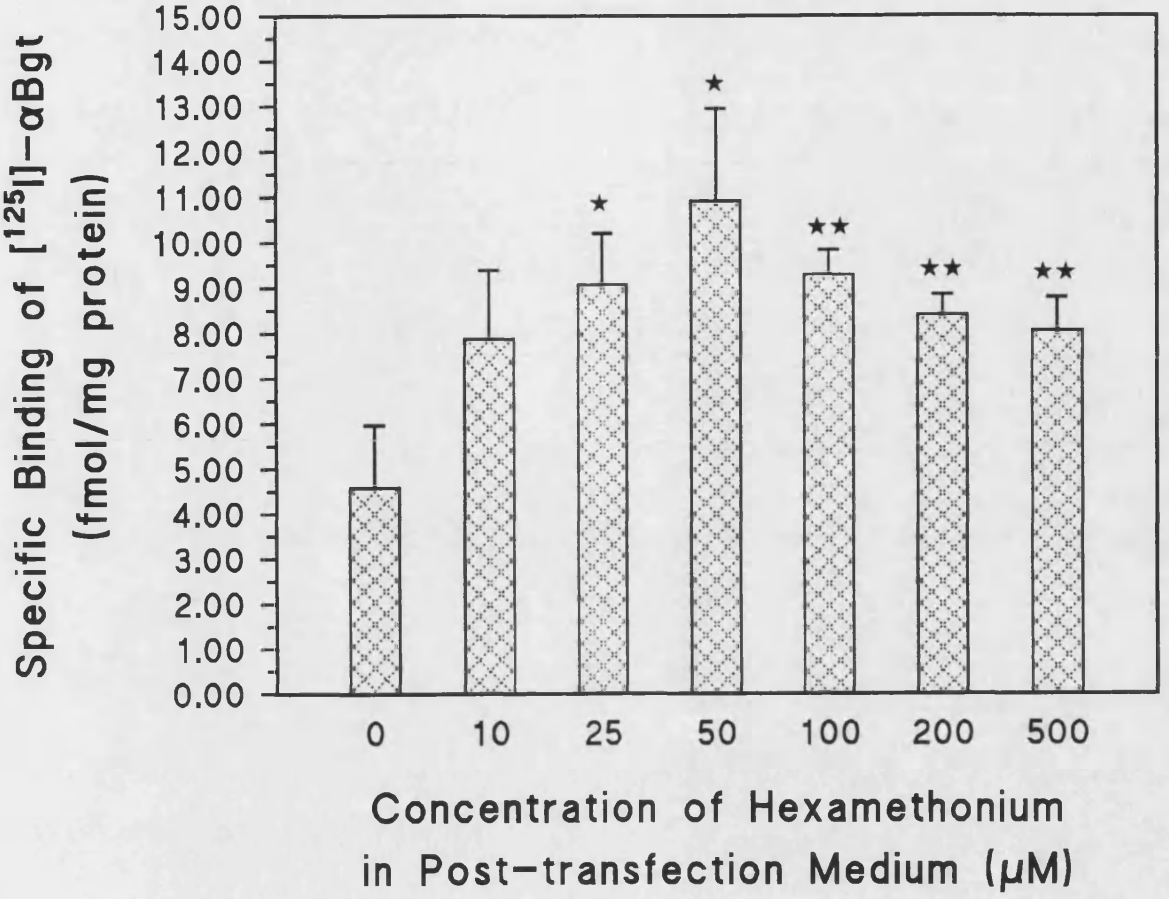
* - Significantly different from cells transfected in the absence of hexamethonium, $P<0.05$

** - No significant difference from cells transfected in the presence of 50μM hexamethonium.

b) The effect of including 10μM dTC in the post-transfection medium, on the specific binding of [^{125}I]-αBgt to COS-7 cells transfected with α7 cDNA. The inclusion of 10μM dTC led to a significant ($P<0.05$) 4 fold increase in the specific binding, over COS-7 cells transfected in the absence of dTC.

In all experiments n=3 and the values are displayed as mean ± σn. Each value within one experiment consisted of 12 replicates.

a)


Specific Binding of [125I]-αBgt
(fmol/mg protein)
15.00
14.00
13.00
12.00
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
10
25
50
100
200
500
Concentration of Hexamethonium
in Post-transfection Medium (μM)

b)

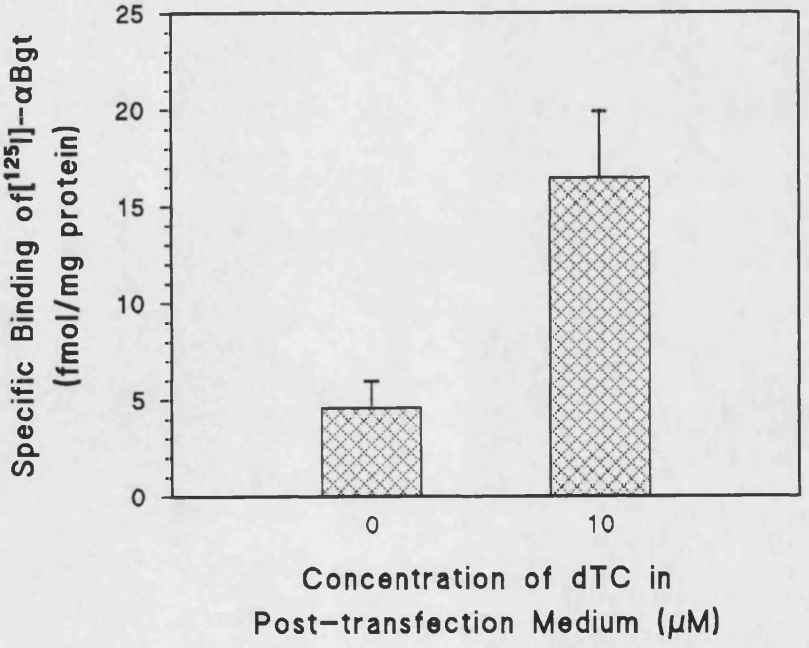

Specific Binding of[125I]-αBgt
(fmol/mg protein)
25
20
15
10
5
0
0
10
Concentration of dTC in
Post-transfection Medium (μM)

Figure 3.7

Comparison of specific binding of [^{125}I]-αBgt between the COS-7 and HEK-293 expression systems transfected under the same conditions. The HEK-293 expression system exhibited significantly ($P<0.02$) greater specific binding under each experimental condition. The table summarises the changes in specific binding within each expression system, relative to cells transfected with α7 cDNA in the absence of antagonist.

In all experiments n=3 and the values are displayed as mean ± σn. Each value within one experiment consisted of 12 replicates.

α7	Cells transfected with α7 cDNA
α7 + dTC	Cells transfected with α7 cDNA and have 10μM dTC included in the post-transfection medium
α7-5HT$_3$	Cells transfected with α7-5HT$_3$ cDNA
α7-5HT$_3$ + dTC	HEK-293 cells transfected with α7-5HT$_3$ and grown under the same conditions of α7 + dTC
Untransfected + dTC	Untransfected cells grown under the same conditions as α7 + dTC
Untransfected	Untransfected cells grown under the same conditions as α7

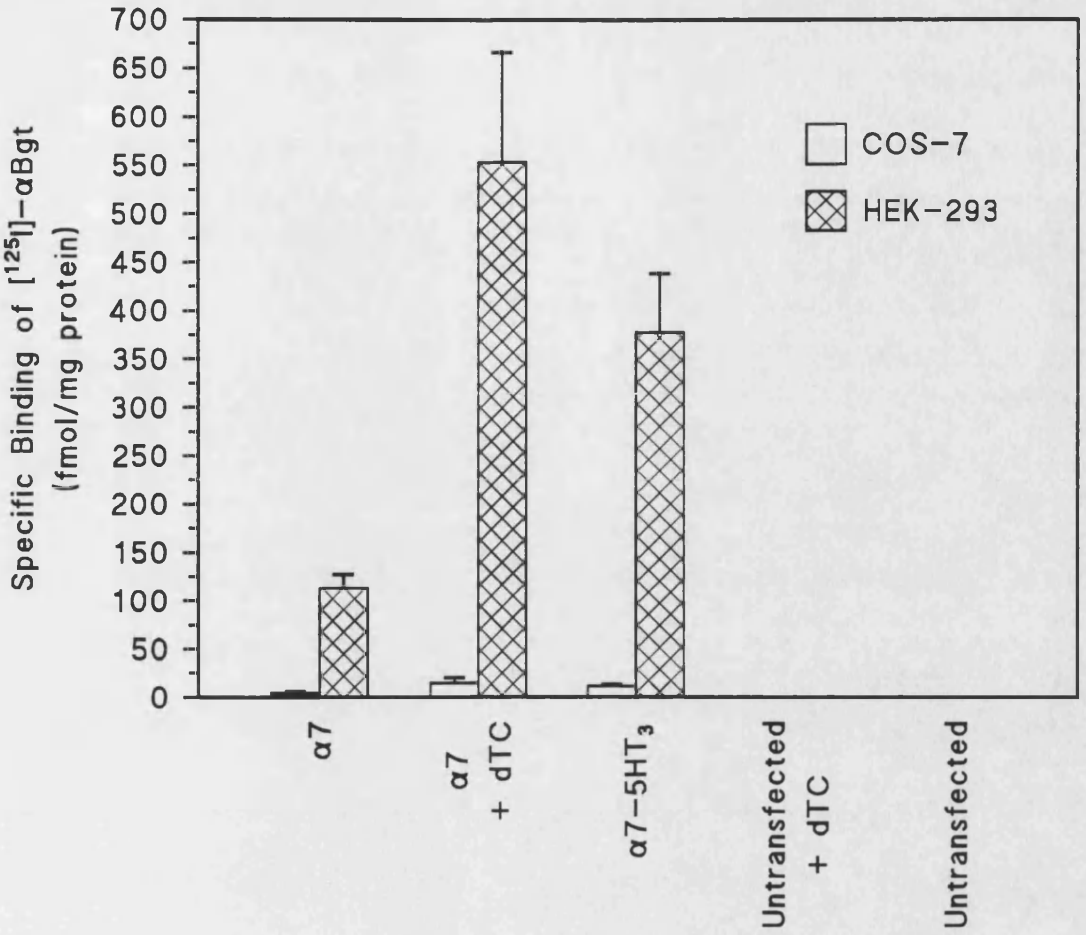


Table of relative specific binding of [^{126}I]-αBgt in the COS-7 and HEK-293 expression systems.

	COS-7	HEK-293
α7	100%	100%
α7 + dTC	324.7% ± 150.0%	489.8% ± 123.0%
α7-5HT$_3$	262.2% ± 31.8%	334.1% ± 65.7%
α7-5HT$_3$ + dTC	----------	345% ± 63.4%
Untransfected	None Detected	None Detected
Untransfected + dTC	None Detected	None Detected

Figure 3.8

Inhibition of specific binding of [^{125}I]-αBgt to COS-7 and HEK-293 cells, transfected with α7 cDNA and with 10μM dTC included in the post-transfection medium, by MLA. The specific binding to transfected cells in the absence of MLA was designated as 100%. The IC_{50} for the α7 nAChR expressed in HEK-293 cells was 0.9±0.1nM. For the COS-7 cells, three curves were calculated. The dotted lines represent curves through the two points either side of a curve, passing through the mean of the two points (solid line). Since there were only two points in that region of the graph, the 'weighting' given to each point could not be determined. Hence, the generation of a curve through the mean of the two points. The IC_{50}'s for each curve was (from left to right) 3.0±0.1nM, 9.9±6.9nM and 16.7±2.8nM respectively. The modified Hill equation used to generate the curves was-:

$$y = 100-(100/1+(k/x))^n$$

$k = IC_{50}$

n = Hill number (this was generated for each curve and was ≅1.4)

In all experiments n=2 and the values are displayed as mean ± σn. Each value within one experiment consisted of 12 replicates.

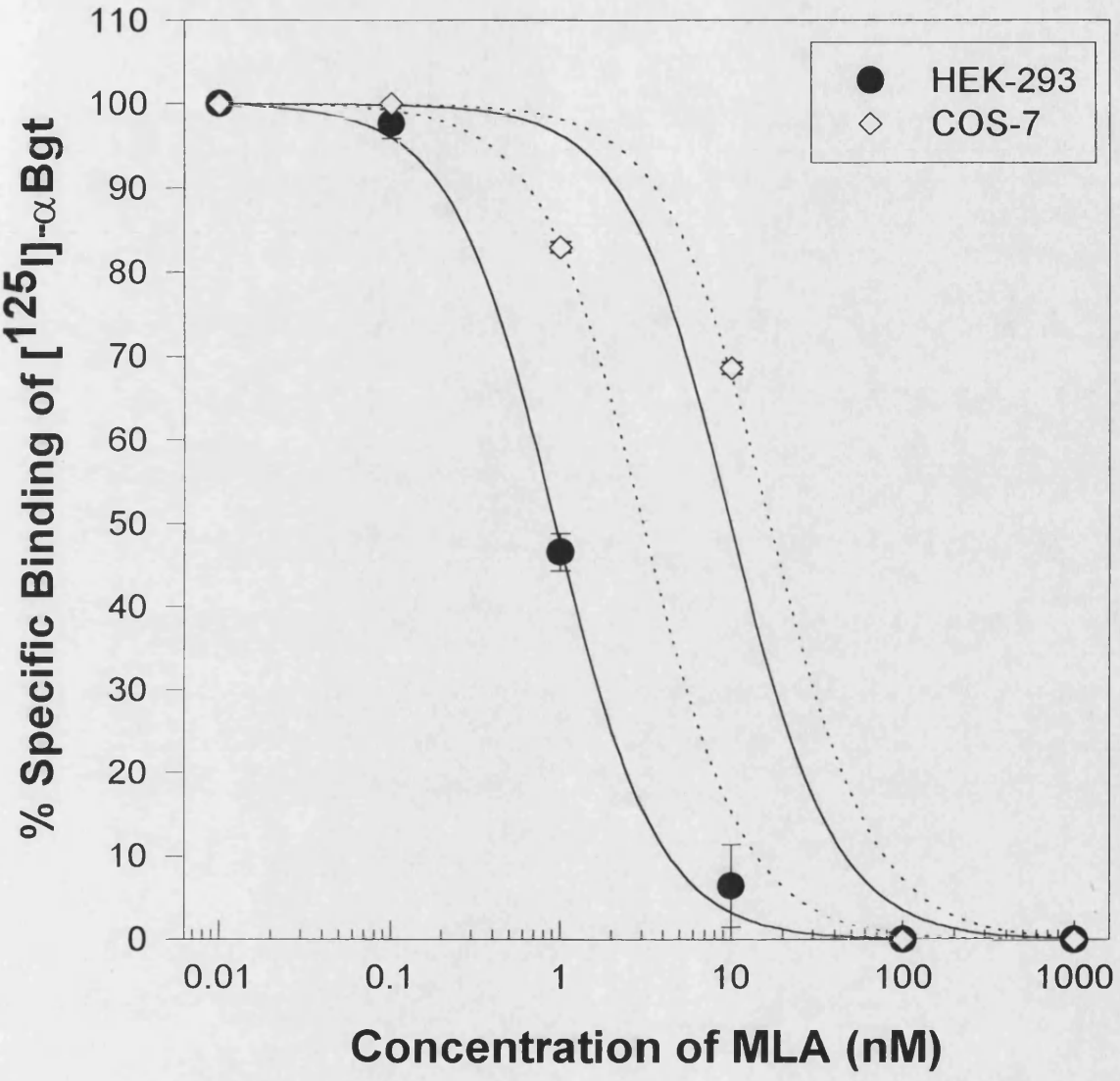

HEK-293
COS-7
% Specific Binding of [125I]-αBgt
Concentration of MLA (nM)
110
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
100
1000

Figure 3.9

Fluorescence labelling of untransfected and transfected COS-7 cells by FITC-αBgt. Total and non-specific binding were performed as detailed in Chapter 2. The upper two panels on each page were viewed under epifluorescence. The lower two panels on each page were viewed under inverted phase contrast (viewing magnification in brackets).

a) Untransfected COS-7 cells grown under same conditions as b (x200)

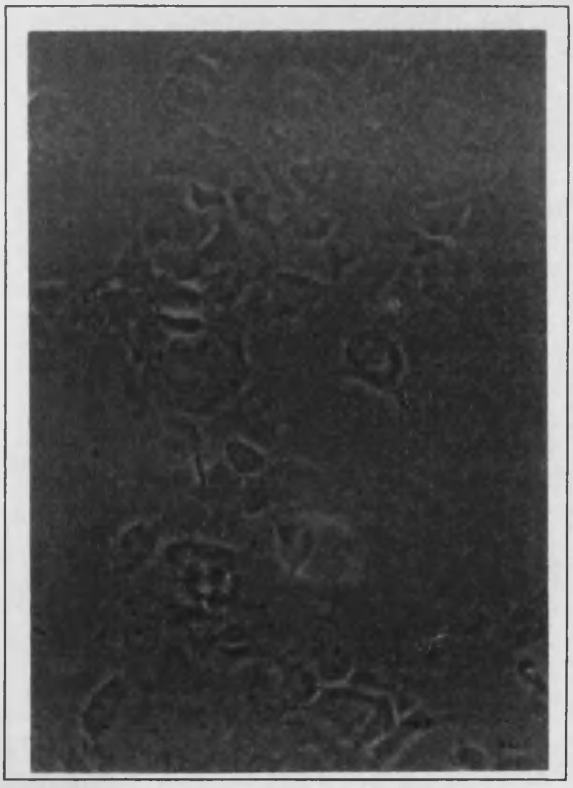
b) COS-7 cells transfected with α7 cDNA (x100)

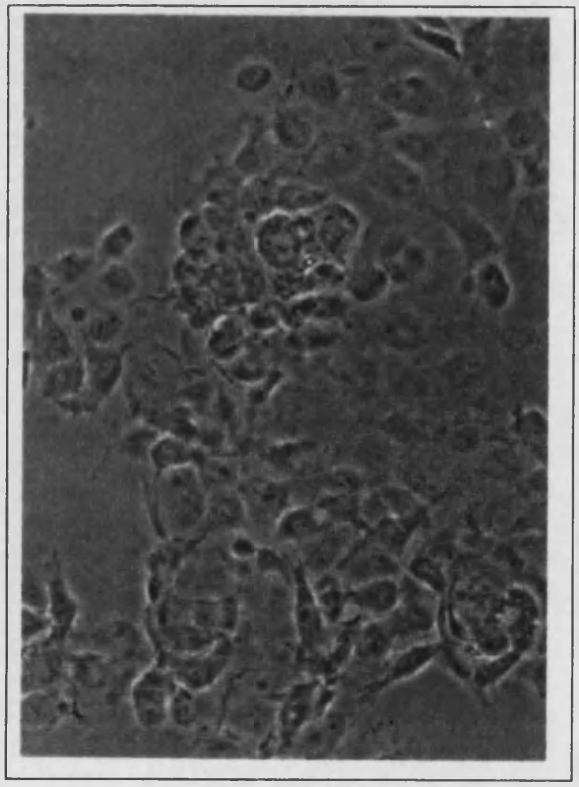
c) COS-7 cells transfected with α7 cDNA with 10μM dTC included in the post-transfection medium (x100)

d) COS-7 cells with α7-5HT_3 cDNA (x100)

a) Untransfected Total Binding

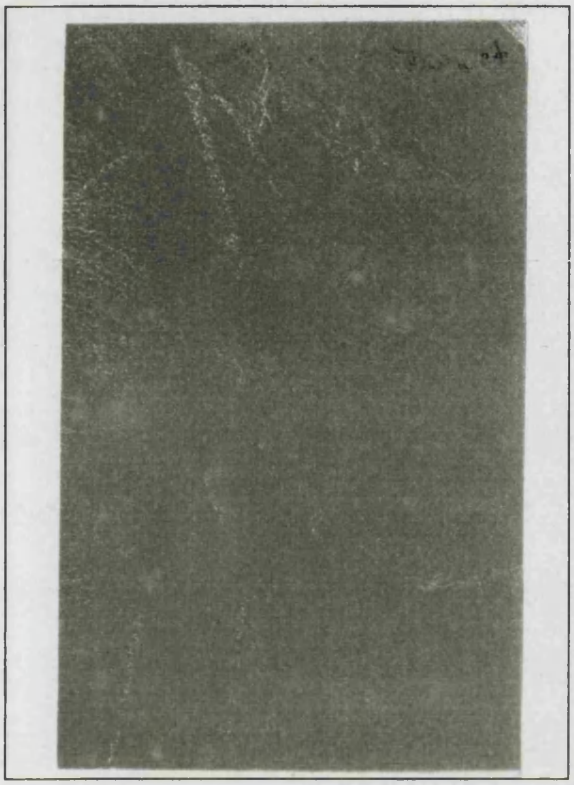
Untransfected Non-specific Binding

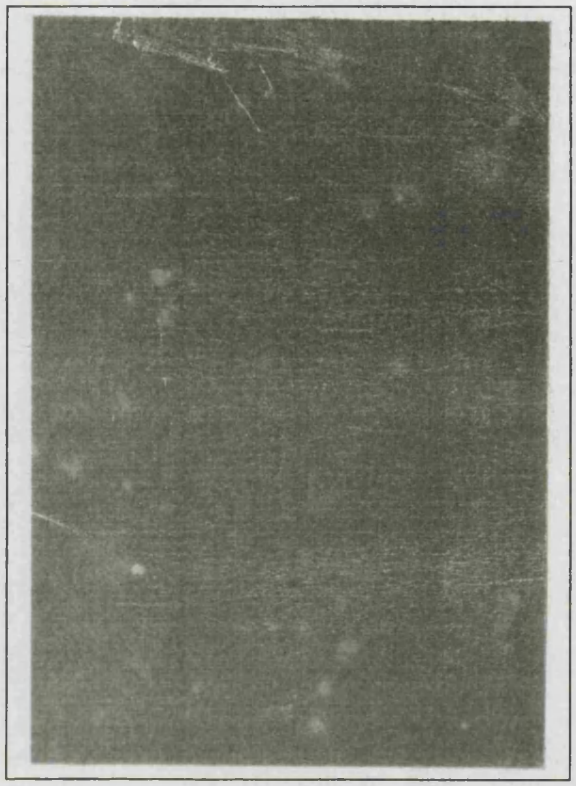


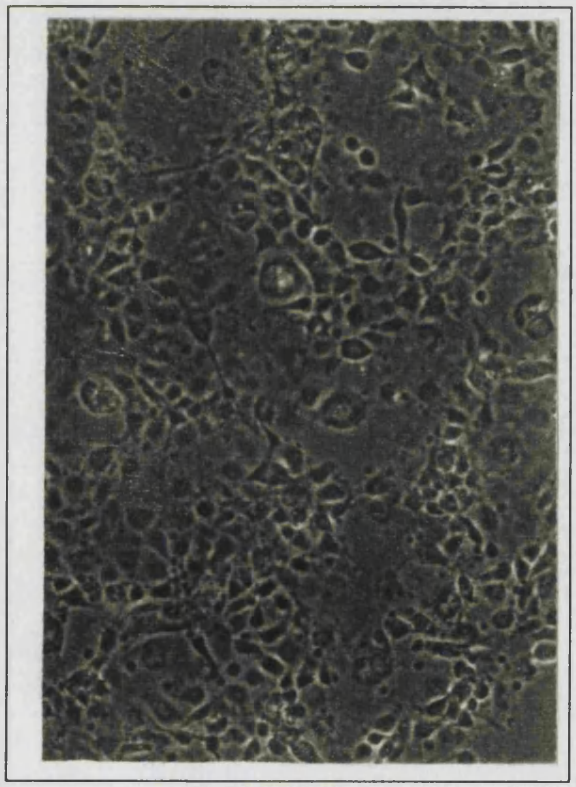


b) α7 Total Binding

α7 Non-specific Binding

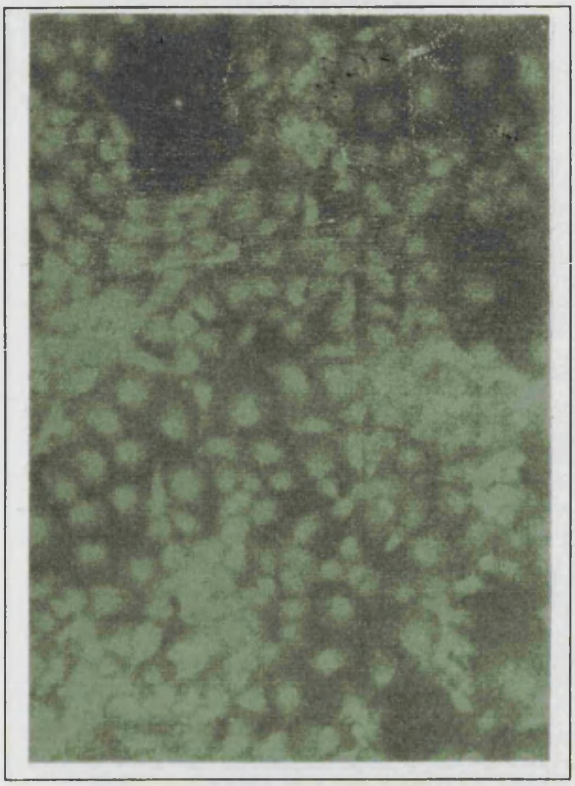


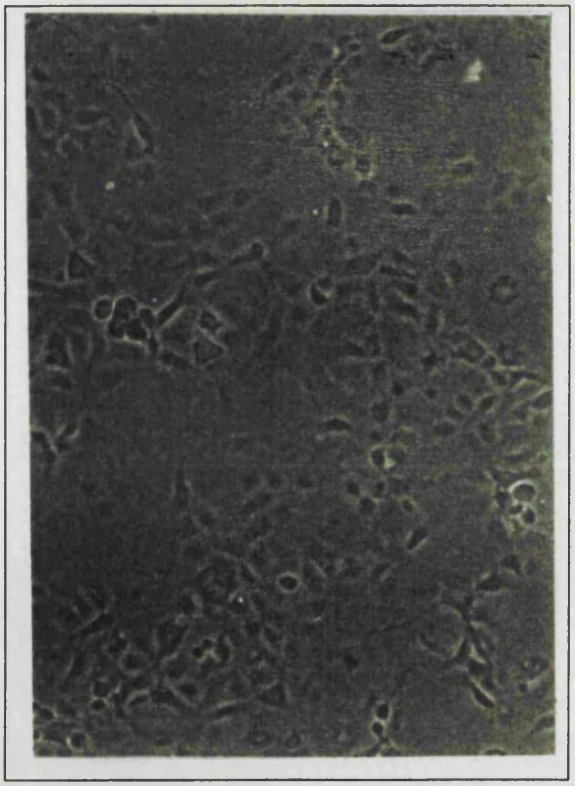


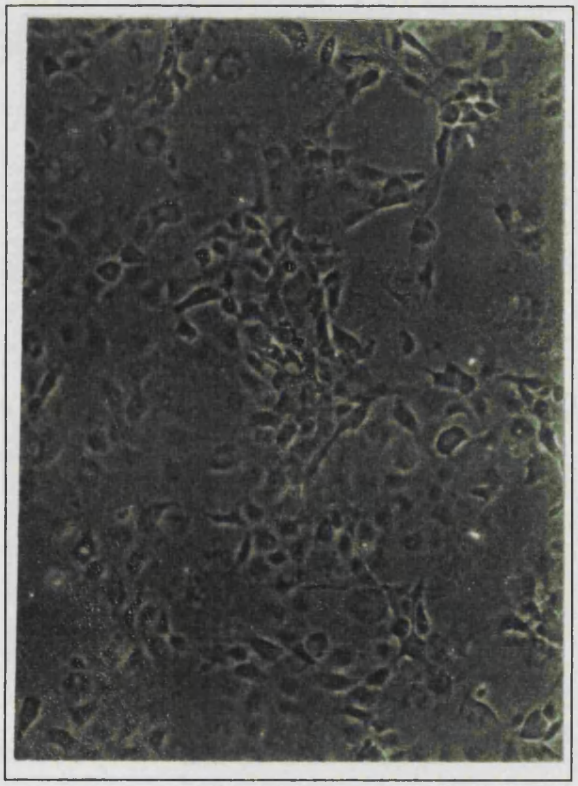


c) α7+ 10μM dTC Total Binding

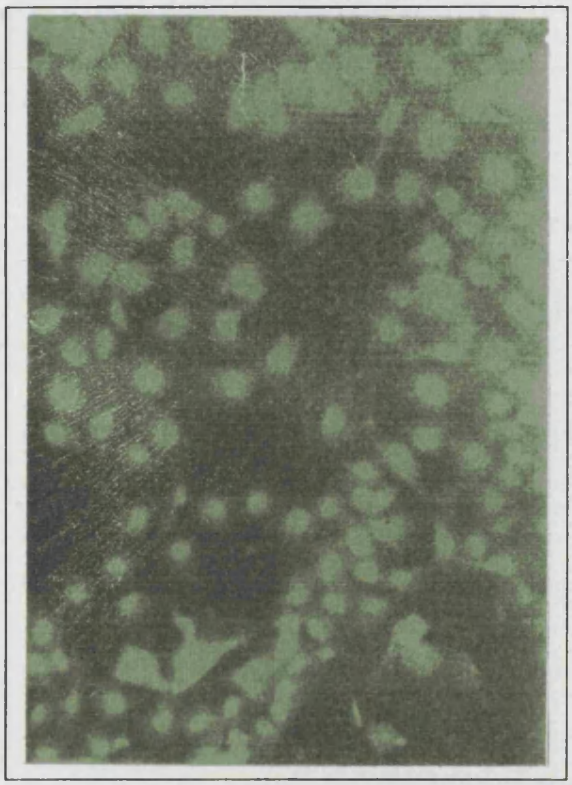
α7+ 10μM dTC Non-specific Binding



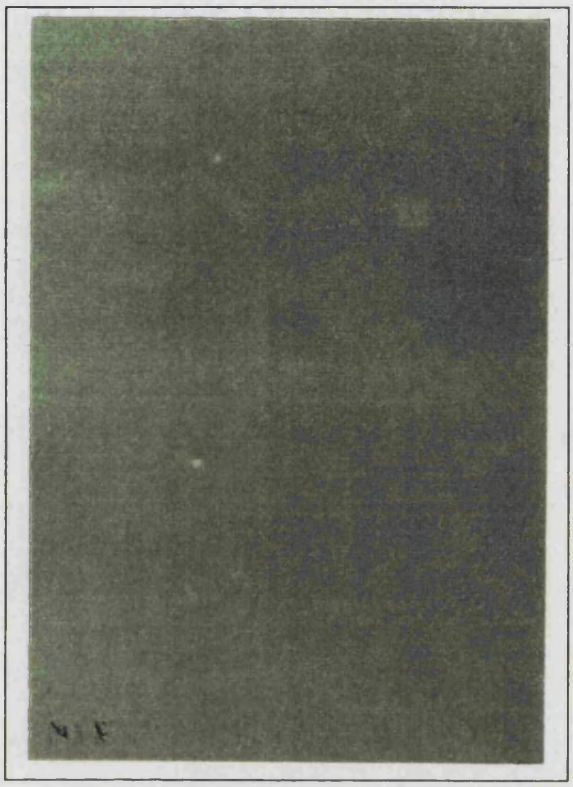


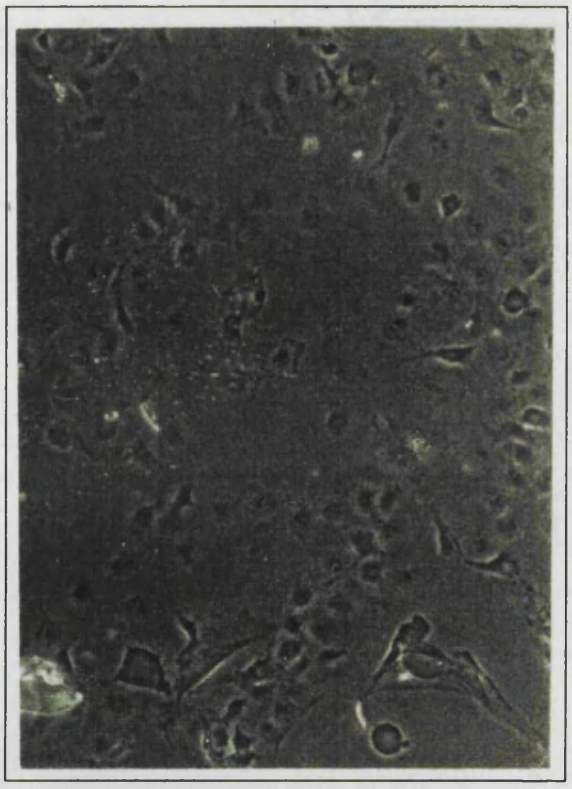


d) α7-5HT$_3$ Total Binding



α7-5HT$_3$ Non-specific Binding





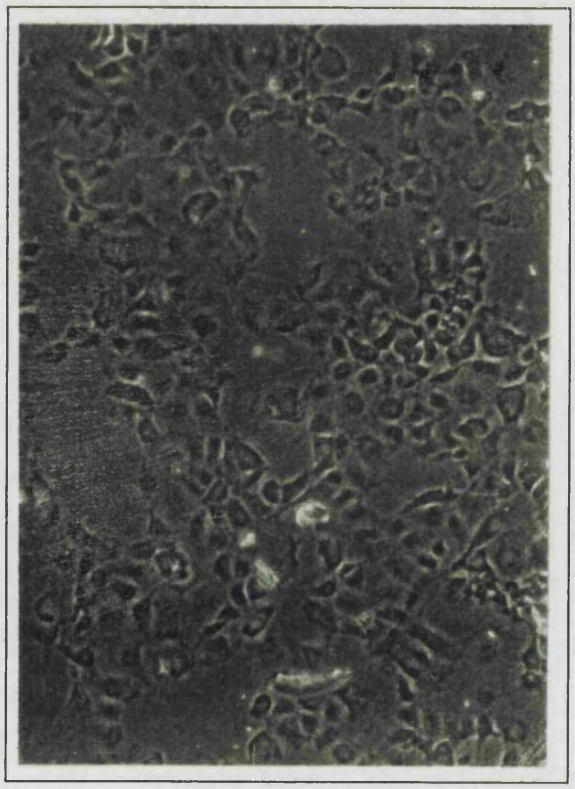


Table 3.1

Inhibition of [^{125}I]-αBgt and [^{3}H]-nicotine binding to nAChRs in different tissues, by MLA. The Cheng-Prusoff equation was used to determine the IC_{50} from the K_i and K_d (Cheng & Prusoff, 1973). Data from Barrantes *et al.*, 1995; Wonnacott *et al.*, 1993; Ward *et al.*, 1990; Macallan *et al.*, 1988.

Source of nAChRs	MLA (IC_{50})
Cultured Hippocampal Cells (αBgt Sites)	15.8×10^{-9}M
Brain P2 Membranes (αBgt Sites)	3.3×10^{-9}M
Hippocampal Cell Membranes (αBgt Sites)	12.0×10^{-9}M
Locust Ganglia (αBgt Sites)	3.6×10^{-9}M
Human Muscle (αBgt Sites)	3.9×10^{-5}M
Human Muscle Cell Line (TE671) (αBgt Sites)	1.3×10^{-4}M
Brain P2 Membranes (Nicotine Sites)	8.3×10^{-6}M
Locust Ganglia (Nicotine Sites)	3.5×10^{-4}M

Table 3.2

Inhibition of [^{125}I]-αBgt and [^{3}H]-nicotine binding to nAChRs in different tissues, by MLA and other related norditerpenoids. The IC_{50} values determined using the Cheng-Prusoff equation on data from Hardick *et al.*, (in press).

	αBgt Binding Sites (IC_{50})		Nicotine Binding Sites (IC_{50})
	Brain	Muscle	Brain
Methyllycaconitine	3.3×10^{-9}M	6.0×10^{-6}M	8.3×10^{-6}M
Lycoctonine	2.0×10^{-5}M	-	$>2 \times 10^{-4}$M
Aconitine	3.8×10^{-5}M	$>2 \times 10^{-4}$M	$>2 \times 10^{-4}$M
AB-Aconitine	6.2×10^{-7}M	$>2 \times 10^{-4}$M	$>2 \times 10^{-4}$M
MSB-Aconitine	12.0×10^{-9}M	8.4×10^{-5}M	$>2 \times 10^{-4}$M

\- Not determined

AB-Aconitine 3-deoxy-18-*O*-desmethyl(2-aminobenzoyl) aconitine

MSB-Aconitine 3-deoxy-18-*O*-desmethyl[2-(methylsuccinimido)benzoyl] aconitine

Chapter 4.

4. Investigation of the Key Residues Involved in the nAChR Subtype Specific Binding of MLA

4.1. Introduction

This chapter will investigate MLA's nAChR subtype specificity, by comparing the MLA sensitivities and amino acid sequences of 3 nAChR α-subunits. From this data point mutations will be introduced into the α7 nAChR cDNA, in order to alter the binding of MLA to the expressed mutant receptor. However, the mutations should not effect the receptor's ability to bind other nicotinic agonists and antagonists. Results of the site-directed mutagenesis and some electrophysiology data from the mutant receptors will be presented. The results will then be discussed in terms of providing information on the key residues involved in the specific binding of MLA.

4.1.1. The Nicotinic Pharmacophore

The notion of a pharmacophore model for nicotinic agonists was introduced in Chapter 1. Sheridan *et al.,* (1986) defined 3 'essential groups' that a pharmacophore, interacting at the ACh binding site, should possess:

A - A cationic centre; Quaternary nitrogen or protonatable nitrogen

B - An electronegative atom; Carbonyl oxygen or aromatic nitrogen

C - An atom to form a dipole with **B**; Carbonyl carbon or delocalising aromatic ring

Their 'Ensemble' method of comparing the molecular structures of conventional nAChR agonists and antagonists, permitted the dimensions of the pharmacophore to be calculated. The distance between groups A & B and A & C were ≅ 4.8Å and 4.0Å respectively. The distance between groups B & C was fixed at 1.2Å, the length of a covalent carbonyl bond.

When the molecular structure of MLA was characterised by proton and ^{13}C mass spectrometry (Jennings *et al.,* 1986), the pharmacophore model was applied to it. Figure 4.1 shows the application of this model to MLA and a selection of other nicotinic ligands. The structure of MLA fitted the independently determined pharmacophore model and also fulfilled the stereochemical criteria for a nicotinic ligand, proposed 16 years before the discovery of its structure (Beer & Reich, 1970; Chothia & Pauling, 1970).

The pharmacophore model predicts the same structure for agonist and antagonists. The model does not distinguish between the binding of an agonist or an antagonist, consequently it does not explain the difference in action of an agonist and an antagonist at the nAChR. Beers & Reich (1970) believed that the differences in agonist stereo-volume may have been, in part, responsible for receptor specificity. It was noted that nAChR antagonists tended to be of a larger stereo-volume when compared to the agonists (cf. structures in Williams *et al.,* 1994). With this in mind, it was proposed that this larger stereo-volume, when docked at the agonist site, could inhibit the nAChR's transition to the activated quaternary form (Sheridan *et al.,* 1986).

This implies the possible involvement of amino acid residues not usually associated with agonist binding.

4.1.2. Criteria for the Choice of Point Mutations

The 'Multiple Loop' model of the ACh binding site was discussed in Chapter 1. Point mutations, synthetic peptide mediated toxin binding and immunological characterisation studies established the 3rd peptide loop (α180-α200) as important in determining nicotinic agonist recognition (Balass *et al.,* 1993; McCormick *et al.,* 1993; Galzi *et al.,* 1991b). This loop contains the previously identified residues Y190, C192, C193 and Y198. Residues Y190 and Y198 have been shown to be involved in binding the quaternary N of nicotinic ligands (Aylwin & White, 1994; Sine *et al.,* 1994). Y190 has been suggested to be the site directly responsible for the anionic/cationic salt bridge interaction between the nAChR, and the quaternary or protonatable N of the nicotinic ligands. Thus it is assumed that this loop also binds the protonatable N of MLA (ie. essential group A in Figure 4.1).

The 3rd peptide loop amino acid sequence of the α7 subunit was aligned and compared with those of the Chick α1 and Locust ARL2 subunits (Figure 4.2). The Chick α1 subunit was isolated from nj/e nAChRs (Barnard *et al.,* 1983). It is responsible for this receptor subtype's sensitivity to nanomolar (nM) concentrations of αBgt and insensitivity to nM concentrations of MLA (Couturier *et al.,* 1990a). The ARL2 α-subunit was isolated from Locust ganglia and forms homomeric nAChRs sensitive to nM concentrations of αBgt

(like homomeric α7 nAChRs). The homomeric ARL2 nAChR is believed to be sensitive to nM concentrations of MLA (M. Amar, personal communication). This assertion is supported by MLA's IC_{50} of 3.6nM at Locust ganglia (ie. where the ARL2 nAChR was isolated from (Table 3.1; Marshall *et al.,* 1988)). Therefore in the 3rd peptide loop of α7 and ARL2 there would be similar amino acids that, by virtue of charge/polarity/size or a combination of these, facilitate the binding of MLA. Conversely in α1, these types of amino acids would be absent. From the sequence alignment, candidate residues were initially chosen on the criteria of charge and polarity (Figure 4.2).

4.2. Results

4.2.1. Design of the Oligodeoxyribonucleotide Primers for the Point Mutations

The α7 nAChR's 3rd peptide loop, with reference to the ARL2 sequence, was pointed mutated to look more α1 like. Three point mutations were chosen (chick α1 numbering of amino acids used):

α7P182 to R (α7PR) Both α7 and ARL2 have the polar imino acid proline (P) at this position. The cis-trans isomerism of P is the rate-limiting step in the folding of many proteins (Brandts *et al.,* 1975). Also this isomerism and P shape, make it an ideal residue for introducing kinks into the secondary structure of a helix or initiating a β-turn (Voet & Voet, 1990). This residue was mutated to the positively charged arginine (R) in α1.

α7K184 to W (α7KW) α7 has a positively charged lysine (K) residue, whereas ARL2 has a negatively charged glutamate (E) at this

position. However, α1 has non-polar tryptophan (W). The polarity of the charge at this position does not seem to affect the MLA binding ability of α7 or ARL2. Replacement K by hydrophobic W in α7 could affect this binding.

α7E187 to W (α7EW) Both α7 and ARL2 have negatively charged E at this position. α1 has W at this position. Using the same rationale as that used for the α7KW mutant, the α7 residue was mutated to the corresponding α1 residue.

Then the oligodeoxyribonucleotide primers were designed and synthesised, for use in PCR mediated site-directed mutagenesis (Figure 4.3).

4.2.2. PCR, Fragment Purification and Cloning

A 'cassette' method was used to insert the mutations in α7. The α7 nAChR cDNA was subcloned from the Flip vector into pBluescript II SK+, as described in Chapter 3. *Eco*RI and *Bgl*II restriction endonuclease sites, lie conveniently to the ends of the region of the receptor to be mutated (Figure 3.1). Hence, wild-type fragments of the α7 nAChR cDNA were 'excised' and mutated fragments 'inserted' using these sites. For each point mutation, PCR production of mutant fragments was carried out using one of the mutation primers and the BR primer. The biotinylated PCR fragments were purified and digested attached to Dynabeads, with *Eco*RI as the first digest and *Bgl*II as the second. The digested mutated fragment was then cloned into an *Eco*RI/*Bgl*II prepared pBluescript II SK+ α7 vector. Clones, from competent cells transformed with the mutant α7 nAChR cDNA, were then sequenced.

4.2.3. Sequencing the Mutants

The proposed mutant clones were sequenced, forward past the *Bgl*II site and reverse past the *Eco*RI site, as described in Chapter 2. For forward sequencing the corresponding mutation primers were used. All reverse sequencing used the Sequencing primer (Figure 4.3). Each sequencing reaction was performed in triplicate and 3 clones were identified (ie. one for each mutant), by reverse sequencing (Figure 4.4). The forward sequencing of each clone produced a sequence identical to the wild-type α7, extending at least 10 base pairs past the *Bgl*II site (results not shown). The 3 mutant α7 clones were each subcloned into the pRc/CMV expression vector, as in Chapter 3. This gave 3 mutant expression clones pRc/CMVα7KW (α7K-W), pRc/CMVα7EW (α7E-W) and pRc/CMVα7PR (α7P-R).

4.2.4. Electrophysiology of the Mutants

The three mutants were to be characterised in the cultured cell expression system, developed in Chapter 3. However, because of the initial low levels of expression detected in the cultured cell expression system and the time taken to develop a solution to this, preliminary characterisation of the mutants was performed in *Xenopus* oocytes. I am indebted to Drs. Amar and Wheeler for carrying out these electrophysiological studies. Wild-type and mutant α7 nAChRs (all using the pRc/CMV expression vector) responses to nicotine and ability to bind MLA, were investigated under voltage-clamp conditions.

The *Xenopus* oocyte expressed homomeric α7 nAChR desensitised very rapidly, when challenged with 100μM nicotine (displaying ≅3.2μA current; Figure 4.5). The EC_{50} for nicotine at this nAChR was 35μM, with a current-voltage curve that inwardly rectifies at more positive potentials (Figures 4.6 & 4.7). These results are similar to those reported by Amar *et al.,* (1993) and Couturier *et al.,* (1990a) for the homomeric α7 nAChR, expressed in *Xenopus* oocytes. This indicates that the pRc/CMV vector does not effect the expression of the α7 nAChR.

No response to 100μM nicotine was seen in *Xenopus* oocytes injected with the α7P-R mutant (S. Wheeler, personal communication). The α7E-W mutant responded to challenge with 10μM, 25μM and 100μM nicotine (Figure 4.8). At 100μM nicotine, the current was equal to that seen in *Xenopus* oocytes expressing the wild-type homomeric α7 nAChR. The above data was included in a dose-response curve to nicotine for the α7E-W mutant nAChR (Figure 4.9). The EC_{50} of 60μM for nicotine was very similar to that for the wild-type homomeric α7 nAChR. No work to determine the α7E-W mutant nAChR's sensitivity to MLA has been performed yet.

Xenopus oocytes injected with the α7K-W mutant produced responses when with 100μM nicotine. However, the magnitude of the current produced was ≅30 times less than that seen for the wild-type or α7E-W mutant (Figure 4.10). Challenge with 500μM nicotine did not appreciably increase the current observed. Figure 4.10 also records the response of this mutant to MLA. Preincubation of the α7K-W mutant with 10nM MLA, did not affect the response elicited by a 100μM nicotine challenge. This would

suggest that the α7K-W mutant nAChR was not blocked by a concentration of MLA proposed to be around its IC_{50}. Unfortunately, this experiment was only performed on one cell.

4.3. Discussion

MLA possesses the 3 essential groups determined by the 'Ensemble' pharmacophore model (Figure 4.1). The validity of the 'Ensemble' method can be tested by comparing the mAChR and nAChR pharmacophores. mAChRs, like nAChRs, binds ACh at its conventional agonist site. Thus, the pharmacophore models and consequently the molecular structures of the ligands of the nAChR and mAChR, should share some similarities (Beers & Reich, 1970). This is aptly demonstrated by the homomeric α9 nAChR, that binds ACh and nicotine, and also the muscarinic ligands atropine and OXO-M (Elgoyhen *et al.,* 1994).

Recognition of the MLA molecule, by the α7 nAChR, is believed to be determined within the 3rd peptide loop (amino acids α180-α200) of the 'Multiple loop' model of the ACh binding site. This is the same region of the α7 nAChR, that has been shown to be involved in binding the nicotinic ligand pharmacophore's essential group A; the quaternary or protonatable N. Although sharing the same pharmacophore, agonists and antagonists have different modes of action at the nAChR. This could be explained by antagonists tending to be sterically larger that agonists. This increased stereo-volume might interact with amino acids, in the ACh binding site, not associated in the binding of agonists. This may also help to explain the

preference of competitive antagonists to bind to, and stabilise the resting or one of the desensitised states of the nAChR (Williams *et al.,* 1994; McCarthy & Stroud, 1989; see Figure 1.4). These residues may be responsible for MLA's nAChR subtype specificity (ie. nM sensitivity at α7 and ARL2 nAChRs and insensitivity at α1 containing nAChRs).

The α7 nAChR amino acids which were to be mutated to the residues present in the α1 nAChR, were chosen by comparing and contrasting the 3rd peptide loop sequences of α7, α1 and ARL2 (Figure 4.2). The mutations should modulate the α7 nAChR's MLA binding, without affecting its ability to recognise nicotine (Figure 4.3). Three mutants were chosen and created by the use of PCR and mismatched primers. These were α7P-R (position α182), α7K-W (position α184) and α7E-W (position α187). The preliminary nature of these data from the mutants should be stressed. Consequently, the conclusions drawn below would still need further investigation.

Wild-type and mutant α7 nAChR responses to nicotine were observed in *Xenopus* oocytes injected with their cDNAs. *Xenopus* oocytes injected with the wild-type α7 nAChR, gave responses to nicotine almost identical to those reported for the homomeric α7 nAChR in the literature. Thus it was assumed that this expression system was investigating homomeric α7 nAChRs also. A previous study on point mutations introduced into the α180-α200 region of the α7 nAChR concluded that they did not affect the receptor's ability to form homomers (Galzi *et al.,* 1991b).

Xenopus oocytes expressing the α7E-W mutant produced a response to nicotine with a similar EC_{50} to that seen for the wild-type nAChR (Figure 4.9). However, the point mutation seemed to produce receptors that desensitised at a slower rate than the wild-type (Figure 4.8). The residue at position α187 is believed to be involved in the conferring αBgt resistance in snake and mongoose nAChRs (Barchan *et al.,* 1992). In mongoose α1, N187 replaces the chick α1 W187, thus introducing a putative glycosylation site. If glycosylated, the carbohydrate 'tree' could sterically hinder the binding of the bulky αBgt peptide. However, the importance of this site is called into question when the snake α1 and human α1 sequences are compared. Both subunits possess S187 at this position, yet snake α1 containing nAChRs are αBgt insensitive and human α1 containing are αBgt sensitive (Barchan *et al.,* 1992; Chaturvedi *et al.,* 1992).

The α7K-W mutant produced nAChRs that had a marked reduction in their response to nicotine, compared to the wild-type (Figure 4.10). The current produced was similar to that reported for the nj/e nAChR in *Xenopus* oocytes (Mishina *et al.,* 1984). The reasons for this are unknown. However, the replacement of an aliphatic charged residue by a more rigid hydrophobic one, may effect the allosteric conveyance of the 'agonist/binding site conformation signal' through the receptor, to activate the channel. A result, seen only in one cell, indicates that the α7K-W mutant is insensitive to 10nM MLA. This result needs further investigation. If repeatable, this data would suggest that α184 may be one of the sites fundamental in determining the nAChR subtype specificity of MLA. It should be noted that in the MLA and

αBgt sensitive α-subunits, α184 is occupied by a charged residue. In MLA insensitive α-subunits, the position is held by hydrophobic or polar residues (Sargent, 1993). Possible predictions could be made about the MLA sensitivities of α8 and α9 subunits, both of which are αBgt sensitive (Elgoyhen *et al.,* 1994; Schoepfer *et al.,* 1990). α8, which has positively charged lysine at position α184, should be MLA sensitive. α9, which has hydrophobic valine at position α184, should be MLA insensitive.

Xenopus oocytes injected with the α7P-R mutant, failed to respond when challenged with nicotine. Helekar *et al.,* (1994) emphasised the importance of prolines being in the correct cis-trans conformation for successful formation of the binding site and surface expression. The loss of the proline was, in positional terms, relatively conservative (ie. the residue replacing the proline (R), was present in the same position in another α-subunit). Thus it seems more probable, as Galzi *et al.,* (1991b) have argued, that the failure to respond was due to the point mutation destroying the ACh binding site conformation. However, another reason possibly lies in the quality of the *Xenopus*' oocytes. Problems were experienced with batches of *Xenopus* oocytes during this study. The remedy, of changing the husbandry of *Xenopus*, came too late for the completion of this study.

However, these preliminary data highlight some of the potential work that could be performed in the cultured cell expression system. Several derivatives based on the norditerpenoid core structures of lycoctonine and aconitine have been synthesised (Blagbrough *et al.,* 1994b; Coates *et al.,* 1994; Hardick *et al.,* 1994). The cultured cells expressing the α7 nAChR

could be used to screen for nicotinic activity in these compounds. The molecular structures of the active compounds could then be used to refine the model of the ACh binding site.

4.3.1. Summary

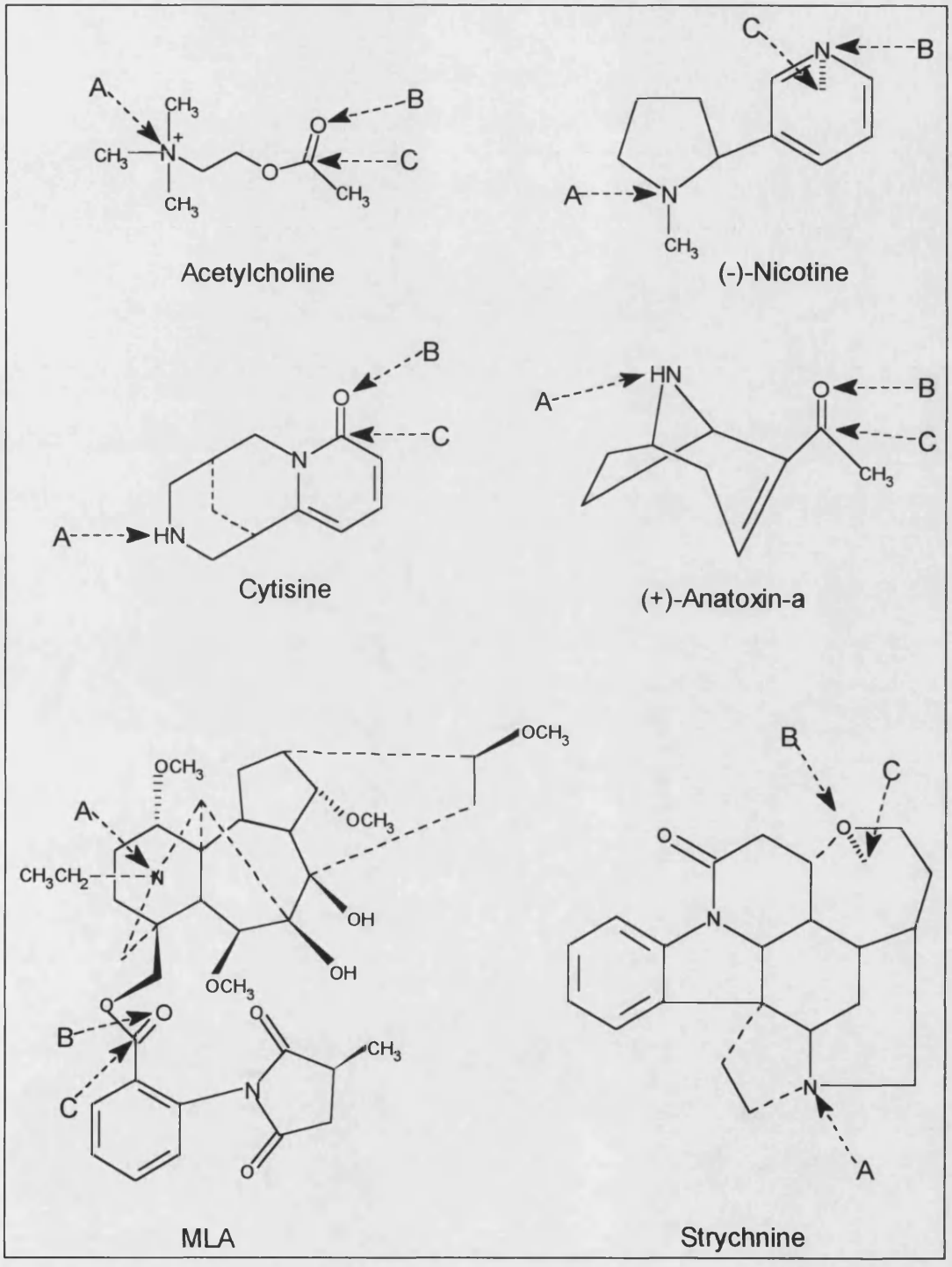
Agonists and antagonists that bind to the ACh binding site on nAChRs, share the same pharmacophore as predicted by the 'Ensemble' model. The 3rd peptide loop, of the proposed 'Multiple loop' model of the ACh binding site, is believed to be important in determining the recognition of nicotinic ligands. The steric size of antagonists may bring into play residues, in the 3rd peptide loop, that are not associated with the binding of agonist, of smaller stereo-volume. Thus, these residues may underpin the nAChR subtype specificity of the competitive antagonist MLA. α7 and ARL2 form homomeric nAChRs sensitive to nM concentrations of MLA and αBgt. α1 containing nAChRs are sensitive to nM concentrations of αBgt, but insensitive to nM concentrations of MLA.

With reference to the ARL2 sequence, three amino acid residues in the α7 nAChR 3rd peptide loop were mutated to the corresponding ones in α1. Preliminary results showed that the α7P-R mutant produced no response to nicotine, which was possibly due to the destruction of the ACh binding site. The α7E-W mutant responded to nicotine in a similar manner to the wild-type homomeric α7 nAChR. The only MLA experiment was carried out on one *Xenopus* oocyte, expressing the α7K-W mutant. This showed the mutant to be insensitive to MLA. If this is a repeatable observation, the

mutation reveals a site in the 3rd peptide loop involved in the determination of MLA sensitivity. This study should be repeated extensively in order to verify the conclusions drawn.

Figure 4.1

Application of the 'Ensemble' pharmacophore model of a nicotinic ligand to the molecular structures of nAChR agonists Acetylcholine, Nicotine, Cytisine and the antagonists MLA and Strychnine. All molecules fitted within the model's parameters. The pharmacophore calculations were not performed on Anatoxin-a. However, the molecule possesses the 3 essential groups (A, B & C) discussed in the text and molecular dimensions similar to acetylcholine (Swanson *et al.,* 1986) (Data from Wonnacott *et al.,* 1983; Ward *et al.,* 1990; Sheridan *et al.,* 1986).


A
CH3
CH3
N+
CH3
O
O
B
C
CH3
Acetylcholine
C
N
B
A
N
CH3
(-)-Nicotine
B
O
C
N
A
HN
Cytisine
HN
A
O
B
C
CH3
(+)-Anatoxin-a
OCH3
OCH3
A
OCH3
CH3CH2
N
OH
OH
OCH3
O
O
B
C
O
CH3
N
O
MLA
B
C
O
O
N
N
A
Strychnine

α7	[177]G	I	P	G	**K**	**R**	T	**E**	S	F	Y	**E**	*C*	*C*	-	**K**	**E**	P	Y	P	**D**[196]
α1	[180]**D**	Y	**R**	G	W	**K**	**H**	W	V	Y	Y	A	*C*	*C*	P	**D**	T	P	Y	L	**D**[200]
ARL2	[189]G	V	P	A	**E**	**R**	**H**	**E**	**K**	Y	Y	P	*C*	*C*	-	A	**E**	P	Y	P	**D**[208]

	Sensitivity		
Subunit	**αBgt (nM)**	**MLA (nM)**	**Nicotine (μM)**
Chick α7	**Yes**	**Yes**	**Yes**
Chick α1	**Yes**	**No**	**Yes**
Locust ARL2	**Yes**	**Yes**	**Yes**

Figure 4.2

Amino acid sequence alignment of 3rd peptide loops from Chick α7, Chick α1 and Locust ARL2. The full α7 and α1 cDNA sequences are 39% and 44% homologous with the full ARL2 sequence, respectively. Sequences were aligned by Cockcroft *et al.*, (1992), using the 'BIOSITE' program. Residues identified in previous studies as being involved in nicotinic ligand binding, are highlighted in *italics*. Charged and polar amino acids are highlighted in **bold** or underlined, respectively. These residues are the potential sites for the point mutations (explained in the text). The table summarises the pharmacological criteria for choosing the α-subunits used.

Figure 4.3

a) Sequences of the oligodeoxyribonucleotide primers used to generate and sequence the mutant α7 nAChRs. Primers α7PR, α7KW and α7EW bind and introduce the point mutations at the beginning of the 3rd peptide loop. The 'Universal M13 Reverse' primer (BR primer) bound to the multiple cloning site of pBluescript II SK+ (≅84 base pairs upstream of the *Bam*HI site). The Sequencing primer bound from nucleotide 876 to nucleotide 858 (see Figure 3.1).

The *Eco*RI site is underlined. The mutated residues and sequences are in **bold.**

b) The molecular structures of the 5 amino acids involved in the point mutations.

a)

α7PR Primer (33mer)

5′-GATTTAGTAGGAATTCGTGGGAAGAGAACTGAG-3′

D L V G I **R** G K R T E

α7KW Primer (36mer)

5′-GATTTAGTAGGAATTCGTGGG**TGG**AGAACTGAGAGC-3′

D L V G I P G **W** R T E S

α7EW Primer (51mer)

5′-GATTTAGTAGGAATTCGTGGGAAGAGAACT**TGG**AGCTTTTATGAGTGCTGT-3′

D L V G I P G K R T **W** S F Y E C C

Biotinylated Reverse (BR) Primer (16mer)

5′-AACAGCTATGACCATG-3′

Sequencing Primer (19mer)

5′-CTGATATCAGTACACAGGG-3′

b)

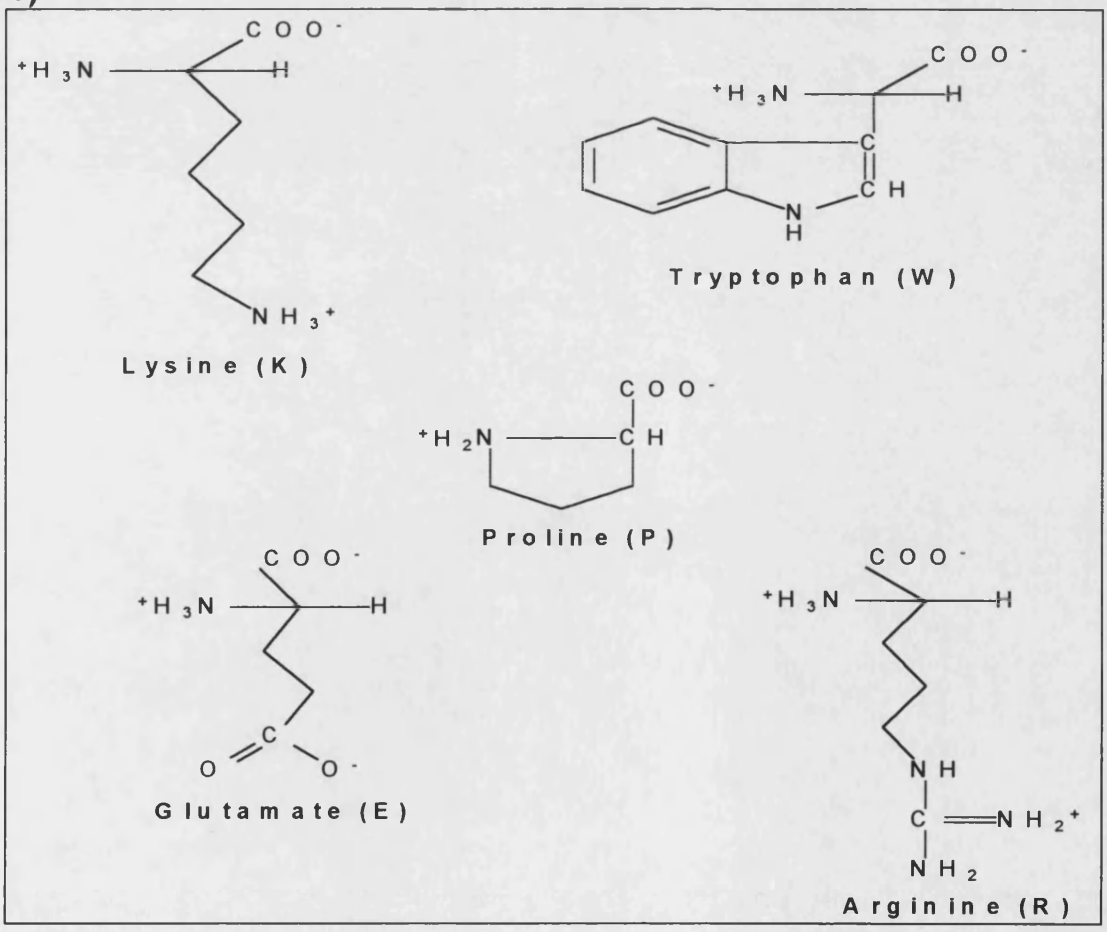


Figure 4.4

The sequences of the mutated 3rd peptide loops in the α7KW, α7EW and α7PR clones. The Sequencing primer sequences the complementary cDNA strands of the mutated receptors. Consequently, the sequence read (ie. read from the bottom to the top of the autoradiograph) will be the complementary of that seen in the α7 cDNA sequence.

The *Eco*RI site is underlined. The mutated residues and sequences are in **bold**.

Mutant α7K-W

K C C E Y F S E T R **W** G
CTTTACAGCACTCATAAAAGCTCTCAGTTCT**CCA**CCC

P I G V L
AG**GAATTC**CTACTAAA
*Eco*RI

Mutant α7E-W

K C C E Y F S **W** T R K G
CTTTACAGCACTCATAAAAGCT**CCA**AGTTCTCTTCCC

P I G V L
AG**GAATTC**CTACTAAA
*Eco*RI

Mutant α7P-R

K C C E Y F S E T R K G
CTTTACAGCACTCATAAAAGCTCTCAGTTCTCTTCCC

R I G V L
ACGAATTCCTACTAAA

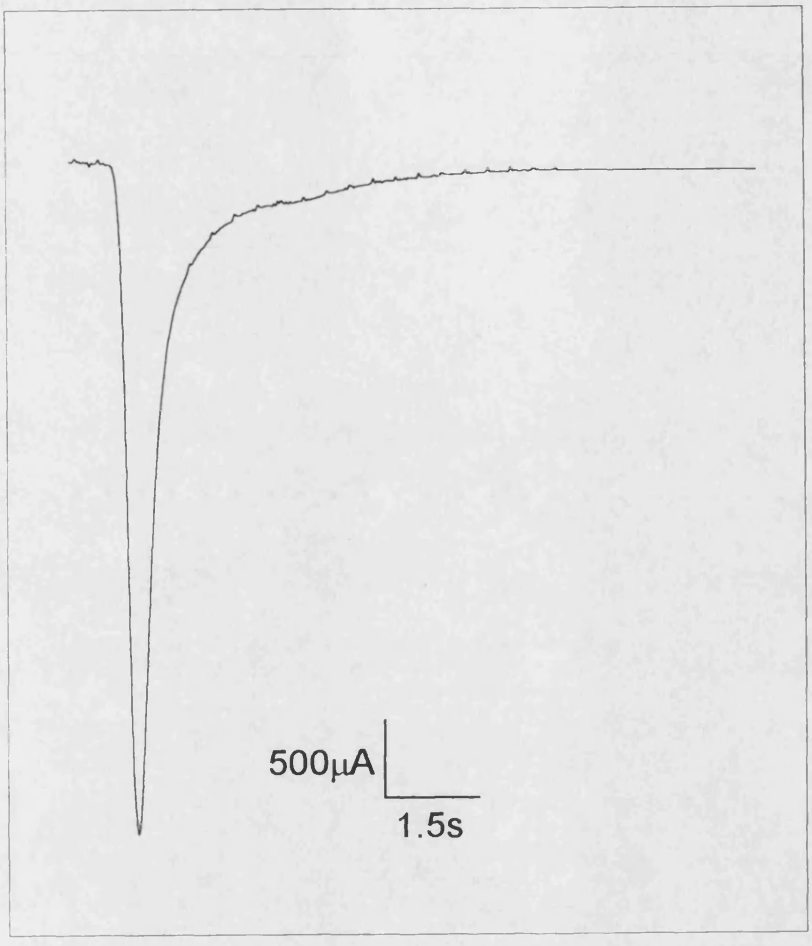


Figure 4.5

Response of the homomeric α7 nAChR, expressed in *Xenopus* oocytes, to 100μM nicotine.

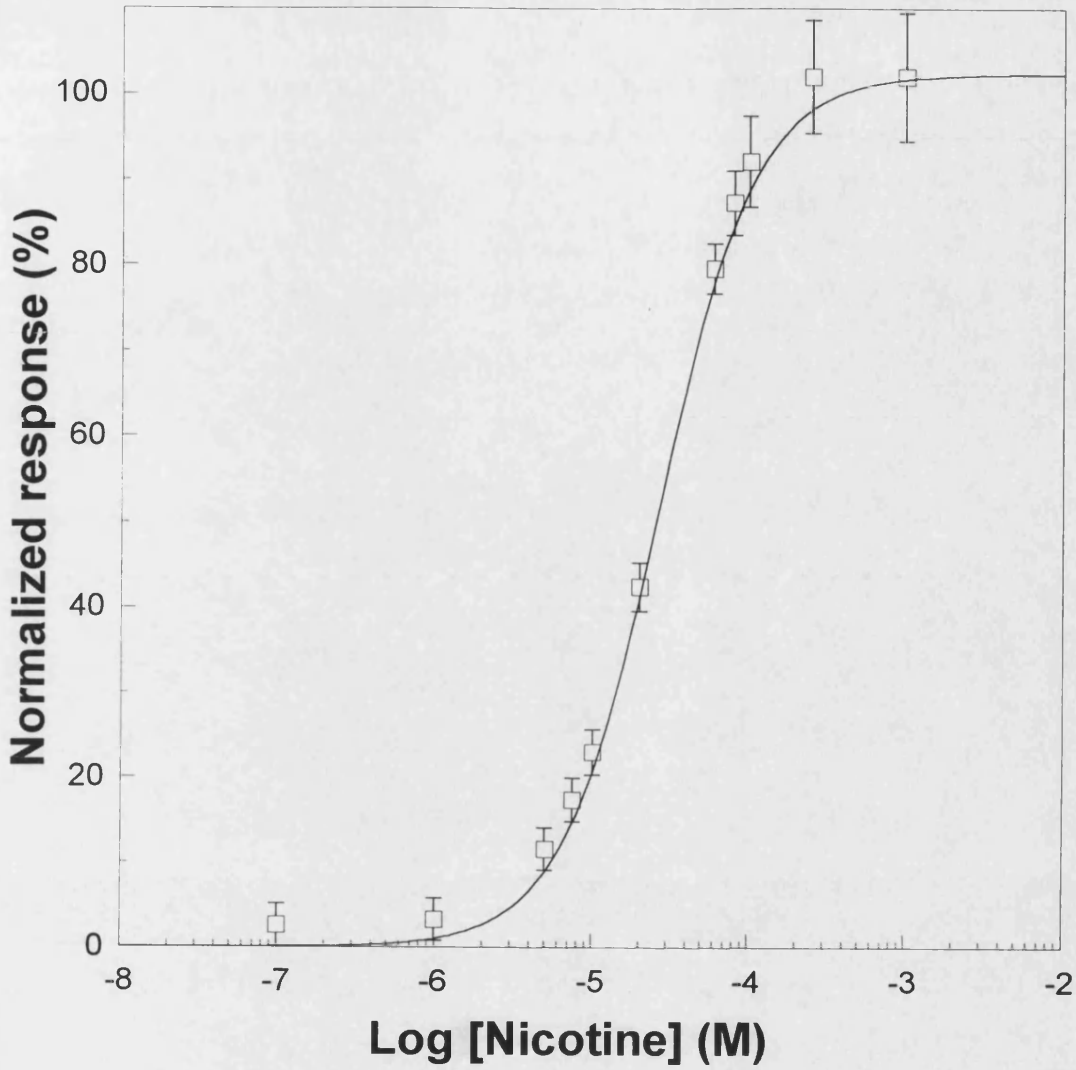


Figure 4.6

Dose response curve to nicotine of homomeric α7 nAChRs expressed in *Xenopus* oocytes. The EC_{50} is 35μM with an Hill number of 1.4. Three cells were used.

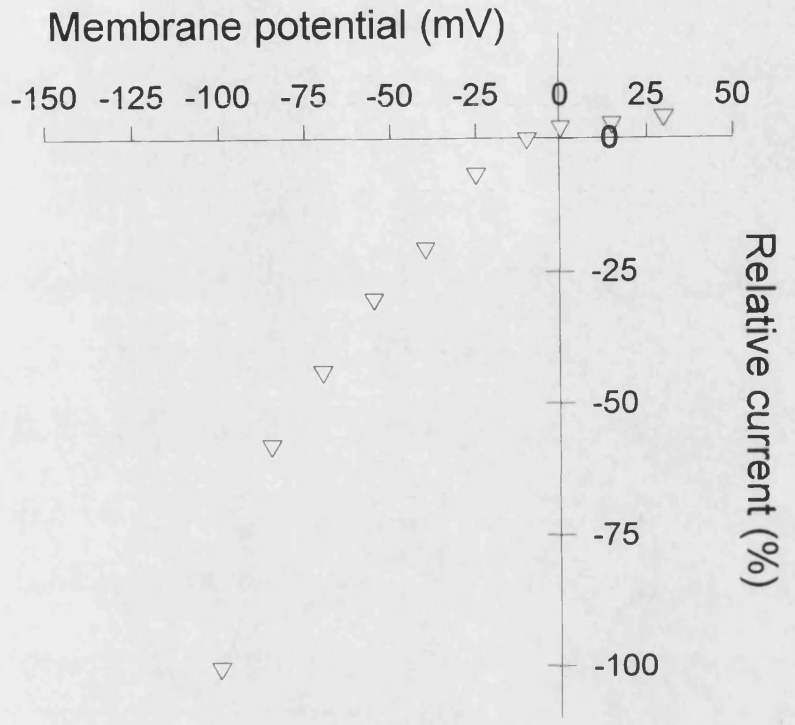


Figure 4.7

The current-voltage relationship of the homomeric α7 nAChR expressed in *Xenopus* oocytes. This illustrates the voltage dependence of nicotine induced currents in the α7 nAChR.

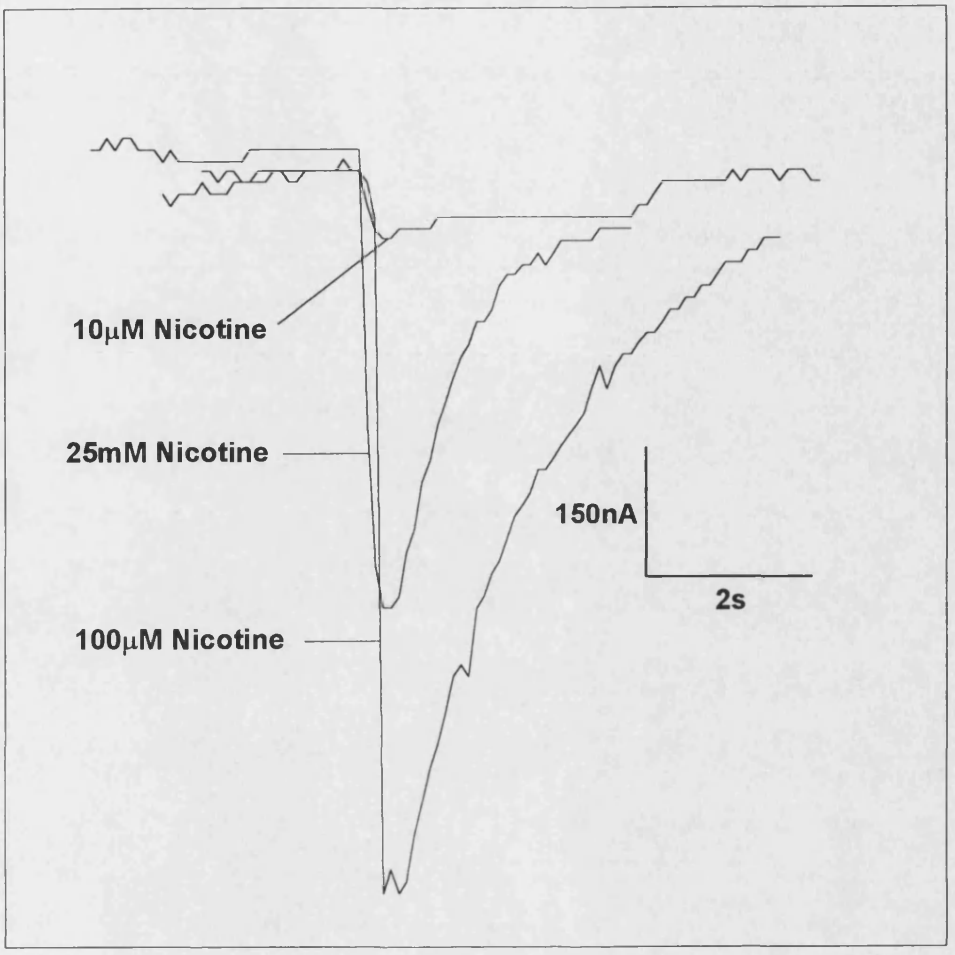


Figure 4.8

Responses of the α7E-W mutant nAChR expressed in *Xenopus* oocytes to 10μM, 25μM and 100μM nicotine.

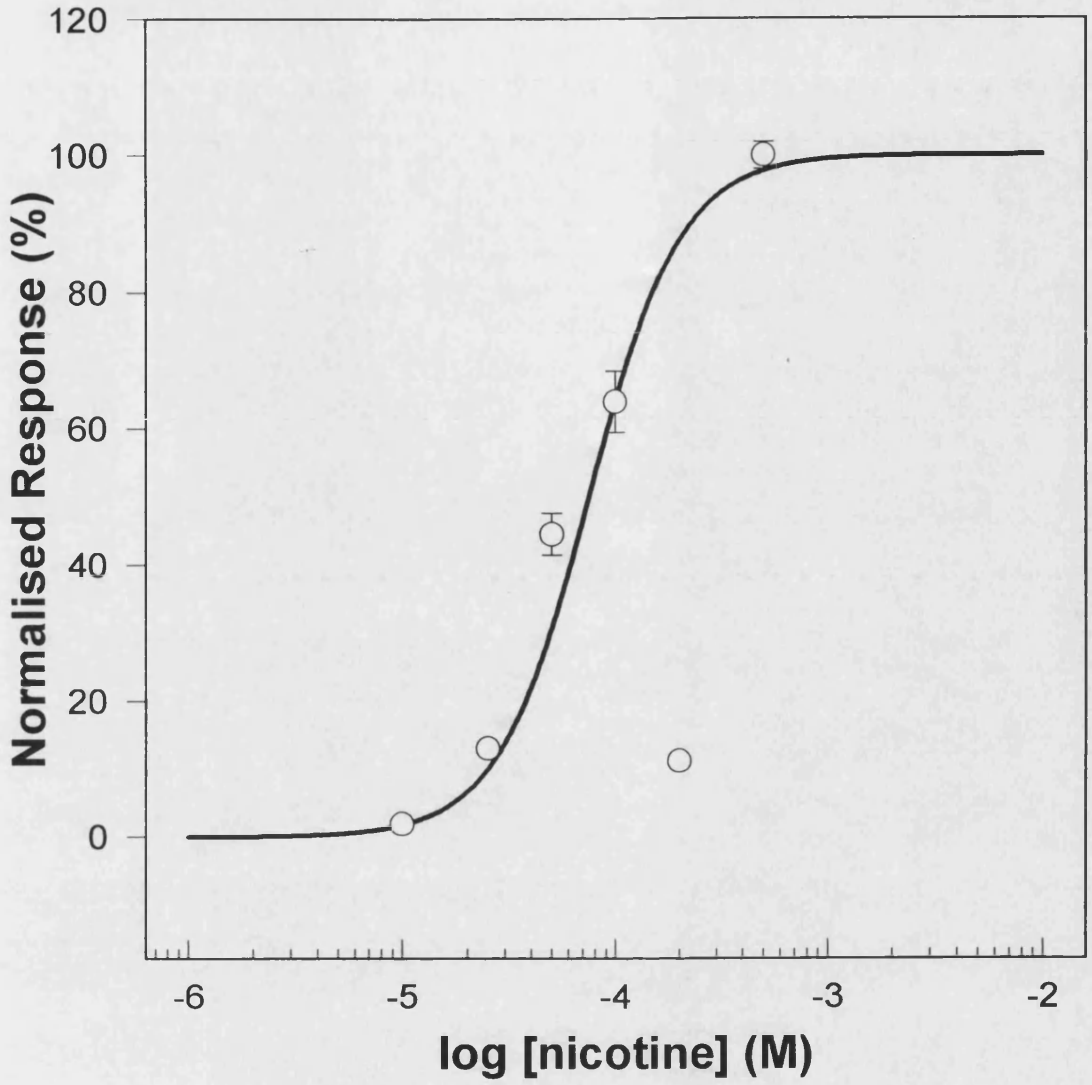


Figure 4.9

Dose response curve to nicotine of the α7E-W mutant nAChR expressed in *Xenopus* oocytes. The EC_{50} is 60μM, with an Hill number of 1.0. Three cells were used.

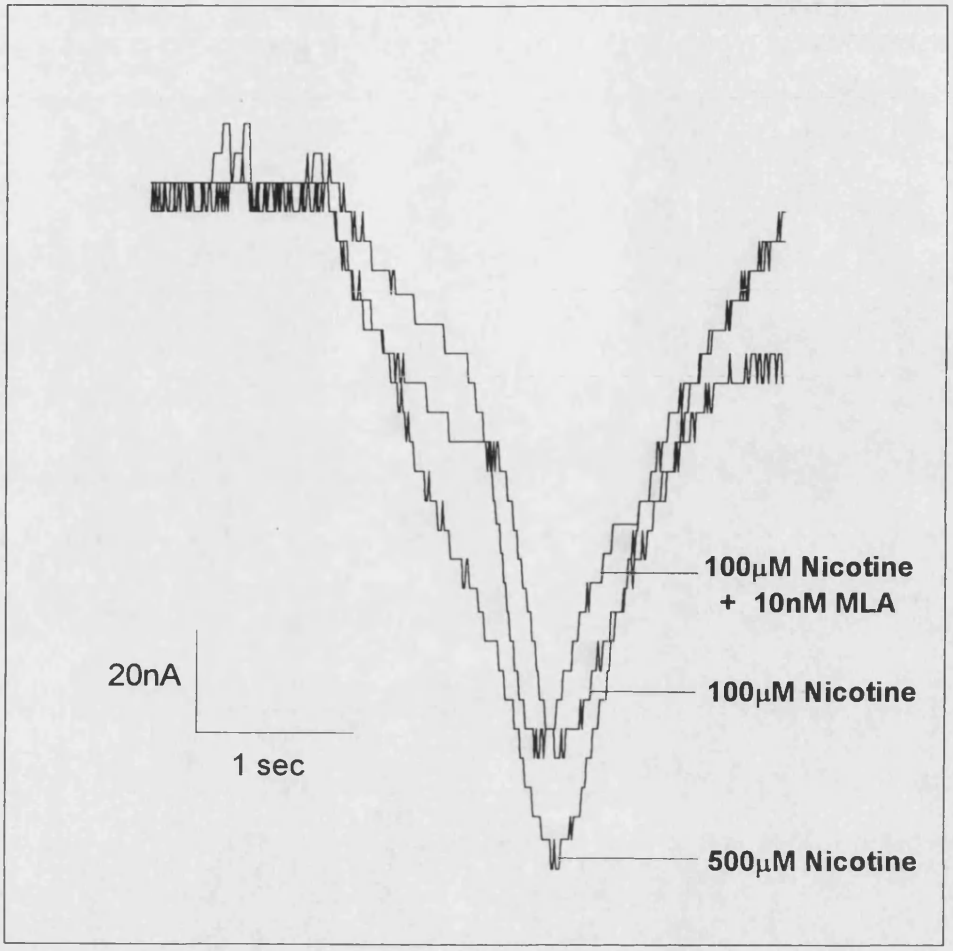


Figure 4.10

Response of the α7K-W mutant nAChR, expressed in *Xenopus* oocytes, to 100μM and 500μM nicotine. The response of this mutant to 100μM nicotine after a 10 minute preincubation with 10nM MLA, was recorded in one cell.

Chapter 5.

5. General Discussion

5.1. Introduction

In 1987, Schuetze and Role said "Compared to muscle nAChR(s), little is known about neuronal nAChRs". Eight years on, the neuronal nAChRs subunits remain, relative to their nj/e counterparts, poorly characterised. However, the application of molecular, immunological and physiological techniques have slowly rectified this. Neuronal nAChR subunits have been isolated from human, chicken, rat, bovine, goldfish, *Drosophila* and locust. (Sargent, 1993; Marshall *et al.,* 1990; Whiting & Lindstrom, 1988). One of the major drawbacks during these studies has been the lack of suitable nAChR receptor subtype specific probes.

αBgt has been used as a standard probe for nj/e nAChRs for many years. The presence of αBgt binding sites in the brain, indicated that the toxin could be possibly used as a probe for neuronal nAChRs. The existence of αBgt binding neuronal nAChRs was called into doubt, when they were found not to coincide with the location of high affinity ACh and nicotine binding sites (Clarke, 1992). Cloning of the α7, α8 and α9-subunits of the neuronal nAChRs and the subsequent electrophysiological studies performed on them, demonstrated that αBgt binding sites were part of functional nAChRs.

A question that then arises is, why have so many subunits contributing to one receptor family? Possible answers to this come from investigating the roles of the neuronal nAChRs. In the brain, the glutamate

receptors constitute the major excitatory transmission system (Hollmann & Heinemann, 1994). nAChRs seem, in several brain systems, to coordinate the release of other neurotransmitters. The $\alpha4\beta2$ nAChR subtype predominates in the brain, and has been implicated in modulating the neuronal release of ACh, GABA, glutamate and dopamine (Beani *et al.,* 1989; Wonnacott, 1989). The involvement of αBgt binding neuronal nAChRs in the expression of neurotrophic factors and neurite retraction, suggests a role for $\alpha7$ containing nAChRs in synaptic maintenance (Freedman *et al.,* 1993; Lipton & Kater, 1989). Rather than having a separate receptor/agonist system for each cellular response, a single tissue may give several different responses to one neurotransmitter, depending on the number of receptor subtypes it expresses. This highlights a 'reductionism' theme, that is extended to the architectural homology of the subunits within the LGIC superfamily (eg cys-loops, Figure 1.2). It is assumed that the receptors within the superfamily function along similar principles. Thus information on the working of one receptor, could be generally applied to the rest of the superfamily. An example of superfamily receptors functioning along similar principles would be the chimaeric $\alpha7$-$5HT_3$ receptor, that possesses nicotinic ligand binding character and an ion channel with serotonergic character (Eisele *et al.,* 1993).

5.2. Expressing the $\alpha7$ Homomeric nAChR

The ability of $\alpha7$ subunits to form a functional homomeric nAChR, make it an ideal model on which to study LGIC function. The

subunits are relatively easy to manipulate and give good responses when expressed in *Xenopus* oocytes. The response of the *Xenopus* oocyte expressed homomeric α7 nAChRs to nicotine (Figure 4.5), is very similar to the Type IA response characterised in whole cell recordings from hippocampus (Alkondon *et al.,* 1994; Alkondon & Albuqueruqe, 1993). This indicates the presence of electrophysiologically similar native α7 nAChRs. However, the evidence does suggest that the native α7 nAChRs are homomeric (Puchacz *et al.,* 1994; Anand *et al.,* 1993a). The rapid desensitisation of the α7 nAChRs indicates a role in responding to acute release of ACh (Couturier *et al.,* 1990a).

The modulation of intracellular Ca^{2+} levels seems to be associated with the action of α7 nAChRs. Permeability to Ca^{2+} is a well characterised property of neuronal nAChRs. α7 nAChRs were shown to be particularly Ca^{2+} permeable, with a large proportion of the response seen in *Xenopus* oocytes, being due to the activation of Ca^{2+} dependent Cl^- channels (Seguela *et al.,* 1993).

The establishment of a stable cell line expressing α7 nAChRs, like the M10 cell line for the α4β2 nAChR (Whiting *et al.,* 1991), would permit a greater critical study of the receptor's biochemical, pharmacological and physiological properties, than would be possible in *Xenopus* oocytes or *in situ*. Puchacz *et al.,* (1994) reported the successful production of a stable cell line expressing the homomeric α7 nAChRs. However, the SH-SY5Y cell line used also expressed other native nAChR subtypes (Lukas *et al.,* 1993; Gould *et al.,* 1992). The use of a 'nAChR naive' cell line means that any nicotinic

character, subsequently exhibited by the transfected cell line, would be as a result of the transfecting cDNA(s)' product(s). Transient expression is sometimes used as a intermediate step in between *Xenopus* oocyte and stable expression of a nAChR (Sine & Claudio, 1991b). The transient expression system allows the optimisation of the receptor's transfection, expression and maintenance conditions prior to stable transfection.

The COS-7 and HEK-293 cell lines, both naive in terms of the specific binding of [^{125}I]-αBgt (see Chapter 3), were transiently transfected with α7 nAChR cDNA. Low levels of receptor expression (ie. as determined by the specific binding of toxin) were detected. This result was compared to the low levels of expression seen in cultured cells, transiently transfected with subunits of the NMDA glutamate receptor. The reason for the low expression was traced to 'Ca^{2+} excitotoxicity' (Rothman & Olney, 1987). Glutamate in the culture medium, led to prolonged opening of NMDA receptor and unregulated influx of Ca^{2+} through the channel. This resulted in low receptor expression and eventually cell death. Ca^{2+} excitotoxicity has also been cited as attributing to ischemic muscle cell death (Leonard & Salpeter, 1979), via a proposed mechanism that involves VDCCs and shares some similarities to that described by Wonnacott *et al.,* (1989) for presynaptic nAChR receptor-mediated neurotransmitter release. For the NMDA receptors, this problem was initially overcome by the inclusion of antagonists in the post-transfection medium.

No conventional nicotinic agonist was identified in the $DMEM_g$ or $DMEM/F12_g$. However, that does not exclude the possibility of the

presence of a 'physostigmine-like' allosteric channel activating ligand (Schrattenholz *et al.,* 1993). Sites on the nAChR for nicotinic ligands other than at the ACh binding site and ion channel, were proposed by Hiedmann *et al.,* (1983). Experimental support for such sites was observed when cells expressing the α7 nAChR and grown in the presence of desensitising concentrations of nicotine, exhibited little if any expression (J-L. Galzi, personal communication). The ability to activate the channel in the presence of desensitising concentrations of agonist, is a property of physostigmine at its binding site (Pereira *et al.,* 1993). However, the physostigmine mediated channel activation was inhibited by the use of a channel blocker (Okonjo *et al.,* 1991).

Channel blocking concentrations of the nicotinic antagonists, hexamethonium and dTC, were included in the post-transfection medium. Both antagonists increased the level of specific toxin binding, presumably due to the increased levels of receptor expression (Figures 3.7 & 3.9). The levels of expression could have been also monitored by the use of a Northern blots, to assay the cellular levels of α7 mRNA or an anti-α7 immuofluorescence assay (Barrantes *et al.,* 1995; Couturier *et al.,* 1990a). Hexamethonium was not as effective as dTC, possibly due to its higher IC_{50} or it becoming cytotoxic at concentrations above 50μM or more likely not being as efficacious a drug as dTC. An alternative explanation for dTC's success at increasing the level of specific toxin binding could be that as well as blocking the channel, dTC induces a conformational change in the nAChR.

This conformational change, along with the presence of the 'allosteric channel activator', could be a trigger that leads to receptor upregulation.

The successful expression of the Ca^{2+} impermeable chimaeric α7-5HT$_3$ receptor, may have indicated that Ca^{2+} influx was possibly responsible for the low expression of the α7 nAChR. However, the transfected cells did not exhibit classical features of cytotoxicity due to Ca^{2+} (ie. radical decreases in viable cell number and total protein present). In transfected cells, Ca^{2+} influx could be monitored by the use of a Ca^{2+}-sensitive dye (such as fura-2) and confocal microscopy. This technique would enable the visual quantification, over time, of the amount of Ca^{2+} entering the cells. It would also permit the comparison of Ca^{2+} levels within cells transfected in the presence or absence of antagonists. The data seems to indicate that the presence of antagonists promotes more efficient surface expression of the α7 nAChR. Consequently, the results seen with transfected cells expressing the chimaeric α7-5HT$_3$ receptor may have been due to the receptor, like the 5HT$_3$ receptor, being more efficiently expressed in cultured cells than the α7 nAChR. Comparison of the relative increases in specific toxin binding seen in each expression system, indicate that the HEK-293 expression was more efficient than the COS-7 expression system.

5.3. MLA, the Pharmacophore and the Mutants

The nicotinic character of the α7 nAChRs expressed in the cultured cells, was confirmed by the potent inhibition of specific toxin binding by nanomolar (nM) concentrations of MLA (Figure 3.8). Although sterically

larger than the agonists ACh and nicotine, MLA fulfils the criteria of the 'Ensemble' pharmacophore model for nicotinic ligands.

The potency of MLA at different nAChR subtypes is probably determined by amino acids not directly involved in binding the pharmacophore's essential groups. Three potential amino acids, in the 3rd peptide loop of the α7 nAChR, were selected and mutated. The mutants were characterised in *Xenopus* oocytes, although the initial intention was to use the cultured cell expression system to accomplish this.

Preliminary data from the α7K-W and α7E-W mutants, showed that they formed functional nAChRs. These data also showed that the α7K-W mutant was insensitive to MLA (discussed in Chapter 4). The failure to detect responses to challenges by nicotine in *Xenopus* oocytes purporting to express the α7P-R mutant, might be due to the production of a non-functional nAChR or poor cell quality. However, these results need to be repeated in order to confirm the conclusions drawn above. The data collected on the mutants so far, indicates that expression in the developed cultured cell system could further investigations into the nature of their ACh binding sites and MLA potency.

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